Free Radical Mediated Oxidative Stress in Plant Cancers

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Susan C Horne

ABSTRACT

This project provides for the first time an in depth examination of oxidative stress and antioxidant protection in plant tissue cultures undergoing various stages of ageing and neoplastic progression towards a cancerous state. Two experimental systems were investigated: (1) *Beta vulgaris* cultures comprising two fully habituated (cancerous), lines, one partially habituated line and a non-habituated cell line; (2) *Glycine max* cultures comprised of two similar aged (15 years) cell lines, one of which has lost the ability to produce pigments. Biochemical profiles of free radical activity and the sequential stages of lipid peroxidation were constructed, together with parallel assessments of primary antioxidants (catalase, peroxidase and superoxidase dismutase and the glutathione-ascorbate couple).

The main findings demonstrated that fully habituated cell lines of *B. vulgaris*, possessed activities and very low levels of primary antioxidants, that were on occasion at the assay limits of detection. In the partially habituated and non-habituated cell lines antioxidant levels were low but not to the same extent. The redox status of the cultures decreased as habituation increased and the lowest GSH/GSSG redox ratio was observed in the fully habituated cell lines. Markers of lipid peroxidation increased inversely to decreased antioxidant protection; all *B. vulgaris* cultures had abnormal cytological manifesting morphological characteristics symptomatic of disturbed metabolism, oxidative stress and loss of totipotency.

In vitro aged callus cultures of *G. max* maintained in tissue culture for approximately fifteen years had lost their embryogenic capacity and one of the cell lines has lost the ability to produce pigments. Activities and levels of key antioxidants in these cell lines were greatly diminished and considerable disruption of

the GSH/GSSG ratio concomitant with increased markers of lipid peroxidation was observed.

This study clearly demonstrates that ageing and habituation implying neoplastic progression to a cancerous state involves oxidative stress concomitant with a major and deleterious disturbance in antioxidant protection. The extent to which oxidative stress occurred increased with the age and the degree of neoplastic progression, such that the partially habituated cell line, was not demonstrably different to the normal *B. vulgaris* cell line. The loss of antioxidant protection associated with ageing and habituation involved the compromise of key antioxidant enzymes (catalase, peroxidase and Cu, Zn-SOD) and GSH/GSSG ratios decreased with age and the degree of habituation. This study concludes with an exploration of the role of oxidative stress in *in vitro* ageing and habituation and considers the importance of maintaining antioxidant status in tissue cultures used in plant biotechnology programmes, and emphasises the use of oxidative stress studies to help understand and ameliorate *in vitro* recalcitrance, the loss of totipotency and somaclonal variation.

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ACKNOWLEDGEMENTS

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DECLARATION

This thesis records the results of experiments carried out by myself in the School of Science and Engineering, University of Abertay Dundee, under the supervision of Dr. D. H. Bremner and Dr. E. E. Benson between October 1999 and March 2003. It is my own composition and has not been previously submitted in part or whole for a higher degree.

Signed.....

Susan C Horne

ABBREVIATIONS

A

Absorbance	ABS
Acquired immune deficiency syndrome	AIDS
Adenosine triphosphate	ATP
5-Adenosylmethione	SAM
Aminolevulinic acid	ALA
Analysis of variance	ANOVA
Ascorbate	ASC

B

Benzylamino purine	BAP
Bovine serum albumin	BSA

C

1-Chloro-2,4-dinitrobenzene	CDNB

D

Degrees Celsius	^{o}C
Dehydroascorbate	DHASC
Deoxyribonucleic acid	DNA
2,4-Dichlorophenoxyacetic acid	2,4-D
Dimethylsulphoxide	DMSO

Deionised water	d. H_2O
5,5-Dithiobis(2-nitrobenzoic acid)	DTNB
Dithiothreitol	DTT

E

Ethanol	ETOH
Ethylenediaminetetraacetic acid	EDTA

F

Fluorescein diacetate	FDA

G

Gas chromatography	GC
Glutathione	GSH
Glutathione reductase	GR
Glutathione S-transferase	GST
Gram	g

H

High performance liquid chromatography	HPLC
Hour	hr
Hydrochloric acid	HCl
Hydrogen peroxide	H_2O_2
4-Hydroxyalkenals	4-HEA
Hydroxyl radical	$HO^{\bullet}$

4-Hydroxy-2-nonenal	4-HNE
Hypersensitive response	HR

I

Infrared spectroscopy	IR
Iron	Fe

J, K L

Jasmonate	JA
Localised acquired resistance	LAR
Liquid nitrogen	LN

M

Malondialdehyde	MDA
Methane	CH_4
Methanesulphonic acid	MSA
Methanol	MeOH
1-Methyl-2-phenylindole	MPI
Microgram	µg
Microlitre	µl
Millilitre	ml
Millimolar	mM
Minute	min

N

Nanometer	nm
Naphthaleneacetic acid	NAA
Nicotinamide adenine dinucleotide diphosphate	NADPH
Nitrotetrazolium blue	NBT

O

Oxidised ascorbate	DHASC
Oxidised glutathione	GSSG

P

Parts per million	ppm
Percentage	%
Plant growth regulators	PGR's
Polyunsaturated fatty acid	PUFA
Programmed cell death	PCD

R

Reactive oxygen species	ROS
Reduced glutathione	GSH
Revolutions per minute	rpm
Ribonuclease	RNAse
Ribonucleic acid	RNA

S

Salicylic acid	SA
Second	sec
Sodium dodecyl sulphate	SDS
Sodium hydroxide	NaOH
Sulphydryl groups	SH
Sulphosalicyclic acid	SSA
Superoxide dismutase	SOD
Superoxide radicals	$O_2^{\bullet-}$

T

Tetraethoxypropane	TEP
Thiobarbituric acid	TBA
Tris-hydrochloride	Tris-HCl
Trichloroacetic acid	TCA

U, V W

Ultraviolet	UV
2-Vinylpyridine	2-VP
Weight per volume	w/v

1.0 CHAPTER ONE: INTRODUCTION

1.1 RESEARCH AIMS

The overall aim of this research project is to investigate the role that oxidative stress plays in *in vitro* ageing and the progression of habituation (plant cancer). The study explores the potential roles of neoplastic progression and ageing in long-term culture, particularly with respect to the implications of both for tissue culture recalcitrance. To achieve these objectives the biochemical and morphological characteristics of two types of culture were examined: (1) dedifferentiated callus cultures of aged *Glycine max* possessing different pigmentations and (2) *Beta vulgaris* cell lines in various stages of neoplastic progression. To ascertain the role of oxidative stress in *in vitro* ageing and neoplastic progression, markers of antioxidant status and free radical mediated damage were evaluated in each cell line. In addition, DNA methylation was also investigated as this process is also associated with changes in patterns of stress and development

1.2 EXPERIMENTAL AIMS

1. To evaluate the morphological differences between cancerous and non-cancerous *B. vulgaris* and aged *G. max* cells in tissue culture.

2. To determine, if any, the differences in activity of key antioxidants and levels of free radical mediated reaction products between cancerous and non-cancerous *B. vulgaris* and aged *G. max* cell lines.

3. To evaluate any significant differences in the extent of DNA methylation in cancerous and non-cancerous *B. vulgaris* cells.

1.3 TOOLS OF INVESTIGATION

1.3.1 *Morphological and biochemical markers*

1. Viability tests using fluorescein diacetate (FDA) staining will be used to assist in the visualisation of the morphological characteristics of the cell lines.
2. Enzymatic and non-enzymatic antioxidant spectrophotometric assays. Specifically for: catalase, peroxidase, superoxide dismutase, glutathione reductase and glutathione S-transferase, and non-enzymatic antioxidants glutathione, ascorbate, and sulphydryl groups. Activities of reactive oxygen species (ROS), hydrogen peroxide and hydroxyl radical activity will be measured using an adapted hydrogen peroxide assay and a gas chromatographic assay based on methane production, utilising free radical trapping by dimethyl sulphoxide (DMSO).
3. Lipid peroxidation and secondary aldehydic oxidation products of poly unsaturated fatty acids determined by spectroscopic and calorimetric assays.
4. Global DNA methylation assessment using high performance liquid chromatography (HPLC).

1.4 *IN VIVO* PLANT GROWTH AND DEVELOPMENT: SETTING THE SCENE FOR *IN VITRO* TISSUE CULTURE

Plant development *in vitro* involves complex interactions between tissue culture manipulations and endogenous plant programming; the following section will consider both components. Plant development *in vivo* is comprised of several different morphological stages dependant on reproductive (sexual and asexual) and vegetative growth. Understanding *in vivo* plant development and stress physiology can greatly assist the plant tissue culturist in applying biotechnological manipulations to the greatest affect. This is particularly the case for understanding in *vitro* recalcitrance and ageing, phenomena that are impeded by the lack of, or loss of, responsiveness to tissue cultures to manipulations.

In vitro plant tissue culture can be defined in terms of differentiated (organised growth) and dedifferentiated growth (unorganised growth). Organised differentiated growth is the most prevalent state *in vivo*, with the exception of diseased and infected plants for which dedifferentiated growth can be a consequence of pathogen attach and disease or, it can be a part of the plant's natural defence systems. Thus, *in vivo*, plants show enormous morphological diversity, for example, in the structure and design of their shoot and root systems. Bushy, non-apical dominant plants have multiple shoots and many leaves, whereas apical dominant plants have single stems with a single or multiple flowers at the end. Plants also vary greatly in lifespan; annuals plants complete a whole life span within one vegetation period, whereas perennial other plants can live up to hundreds of years. These inherent qualities can also impact *in vitro* growth patterns.

The vegetative development of plants *in vivo* and *in vitro* depends on the production of new cells from the meristems and secondly the position of the organ's primordial and secondary meristems. Meristems are the regions in plants that differentiate to form new cells and tissues, they are located in the shoot and root tips and, in these positions are referred to as the apical meristems. Reproductive plant development via sexual routes begins with a double fertilisation event where the two sperm nuclei fuse with the egg cell and central cell nuclei (Harada, 1999). The fertilised zygote then undergoes an orderly progression of cell divisions and embryogenesis begins. Embryogenesis establishes the axis of the plant forming a root apical at one end and a shoot one at the other (Wolpert, 2001) , this occurs in three steps, which are often referred to as the globular, heart and torpedo stages (see Figure 1.1).

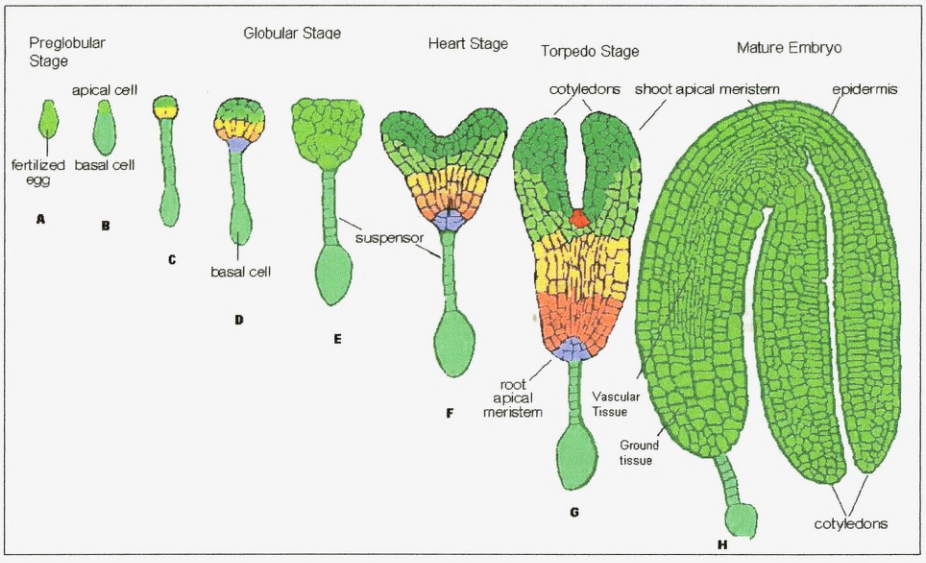

Figure 1.1: Stages of embryogenesis in a dicot plant: (A) Single celled zygote. (B) Zygote divides asymmetrically producing a basal cell that becomes part of the root apical system and apical cell that gives rise to the apical and central regions, which forms the majority of the seedling and becomes the main shoot meristem. (C-F) During the globular stages the eight-celled embryo divides and undergoes rapid organised cell division producing a heart shaped embryo, giving rise to two cotyledons (seed leaves), during this stage the apical meristems are developed and the axial polarity is established. (G) The torpedo stage this is where the embryo elongates and the two coyledons are more differentiated and distinctive. (H) The mature embryo consists of five main structures found along the length of the apical/basal embryo. The apical shoot meritem, the cotyledons, the hypocotyls, the root and the apical root meristem. Image adapted from Wolpert, Lewis. (2001) Principles of Development. Oxford University Press, NY, copyright pending.

The embryo develops inside a seed coat and then, dependant on the species and if it is from temperate regions, the seed can enter a dormancy period (most tropical species do not have a endogenously programmed dormancy period). Dormancy is associated with a decrease in RNA and protein synthesis and during this stage the seed loses almost all its water. The stage before plantlet formation is seed germination this is where the dormancy is broken and RNA and protein synthesis is resumed and the embryo continues on its normal developmental course forming a seedling and eventually a mature plantlet. In the case of *in vitro* plants, zygotic embryos and their associated structures can be used as a source of explants for culture initiation. Furthermore, it is possible to evoke the process of somatic embryogenesis, a process by which embryos are produced from somatic cells through tissue culture manipulations.

At the seedling stage of plant development the primary root is the growing lateral root, during early root formation secondary root meristems are formed at the tip of the lateral root primordium. The secondary root meristem is organised in the same way as the primary root apical, its purpose is to add new tiers to the existing primary root. The primary shoot system is the source of most normal shoot-associated structures. There are some exceptions and particularly *in vitro*, when shoots can arise from sites that are not of the original pre-determined and programmed point of development. These are called adventitious shoots and their consideration is very important in plant biotechnology as they can produce genetically altered plants, as their stability may be compromised (Harding, 2004). The shoot meristems serve two main purposes, the formation of organ primordial and differentiated tissues for the stem and secondly the replenishment of their own meristem-cell pool. If the apical shoot meristem produces

a flower, then the shoot meristem in converted into an indeterminate inflorescences meristem, which produces a flower instead of a leaf, see Figure 1.2 for a schematic overview.

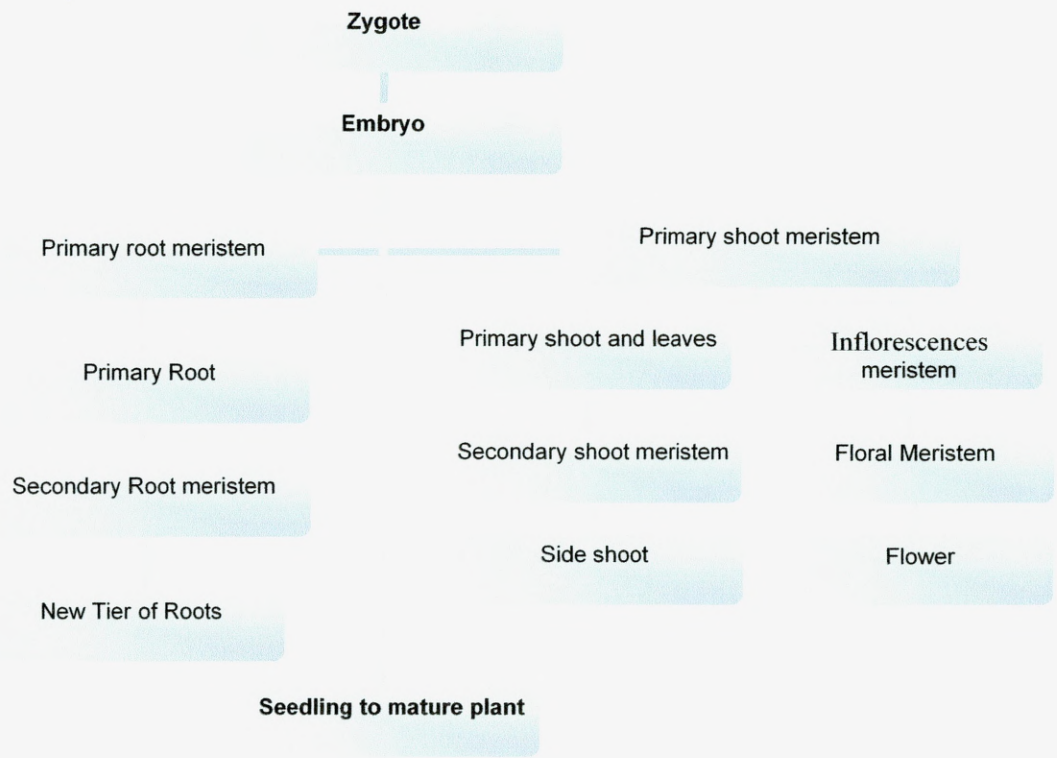

Figure 1.2: Schematic overview of the progression from fertilisation through to secondary embryogenesis and plantlet development. Reprinted with permission from Mechanisms in Development, (Jurgens, 2003), with permission from Elsevier.

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Plant development occurs in response to a facilitated sequential "readout" of predetermined DNA bases. Development growth takes place through the production of cells in the meristem regions. The growth of plants is impacted by environmental cues, roots grow downwards (gravitational cue), shoots grow upwards (photosensitive cue) and leafs shoots and flowers are formed at specific sites, however hormones primarily mediate plant development (Fosket, 1994 and Kende and Zeevaart, 1997).

Plant or phyto hormones naturally occur in plants (Hartmen *et al.*, 2002), they have relatively low molecular weights and are very active in low concentrations and are usually synthesised at a specific site or point of origin and are translocated to other parts of the plant after they are activated by complex cell signalling mechanisms. There at five naturally occurring types of plant hormones, each with a specific role in development (see Table 1.1). However there are also a wide range of chemical components, which can mimic the properties of naturally occurring hormones and the classification of hormones is increasingly more complex.

PGR group	Natural PGR	Formula	Effect on plant development
Auxins	Indole-3-acetic acid	$C_{10}H_9NO_2$	Auxins have the ability to induce cell elongation in stems and facilitate phototropism
Cytokinins	Zeatin	$C_{10}H_{13}NO_2$	Cytokinins stimulate cellular division by speeding the transition of cells from the G2 phase to the M phase of the cell cycle
Gibberellins	Gibberellic acid	$C_{19}H_{22}O_6$	Gibberellins enhance growth, delay flowering and break dormancy. There are over 50 endogenous forms in plants
Abscisic acid	Abscisic acid	$C_{15}H_{20}O_4$	Abscisic acid (ABA) is involved in the closure of stomata, bud and seed dormancy and is known to inhibit other PGR actions.
Ethylene	Ethylene	C_2H_4	A gaseous PGR involved in fruit ripening, floral initiation, breaking dormancy and abscission of plant parts.

Table 1.1: The five types of naturally occurring plant growth hormones, examples of each, the chemical formula and examples of the role they play in plant development.

In addition to natural plant hormones, there are a number of plant growth regulators many of which have been synthesised chemically to produce analogues that mimic the action of naturally occurring plant hormones; many were, originally developed as herbicides. Some of which, such as 2,4-dichorophenoxy acetic acid (2,4-D) have been successfully applied to plant tissue cultures. In such cases these are usually referred to as plant growth regulators (PGRs). In addition there exist naturally occurring compounds that have the ability to influence growth and development, usually in differential ways and these are referred to as plant growth factors. Examples include polyamines, vitamins, inositol and even some micronutrients. The boundaries of definition across these different types of growth mediators are becomingly increasingly contentious. However, all categories are very important to the plant tissue culturist.

In *in vitro* tissue culture the two most commonly used synthetic PGRs belong to the auxin and cytokinin groups. There are a number of synthetic auxins, including 2, 4-D and naphthalene acetic acid (NAA), which are aromatic compounds with carboxylic side chains. Synthetic cytokinins include benzyl adenine purine (BAP) and kinetin. Auxins promote both cell division and cell growth; however the only naturally occurring auxin, indole acetic acid, is sensitive to both light and heat, which greatly limits its use in routine tissue culture manipulations. 2, 4-Dichlorophenoxyacetic acid is the most common synthetic auxin and in most cases is as potent as naturally occurring indole acetic acid (IAA). 2, 4-Dichlorophenoxyacetic acid has been the auxin of choice for many years (Michler and Bauer, 1991, Shigeta and Sato, 1994 and Guis *et al.*, 1997) and is still used frequently today to promote somatic embryogenesis

and organogenesis (Devi *et al.*, 2004, Arunyanart and Chaitrayagan, 2005 and Slater *et al.*, 2003).

Auxins and cytokinins are often applied together in concentrations dependent on the desired effect; a high auxin to cytokinin ratio promotes root growth and a high cytokinin to auxin ratio promotes shoot growth. An intermediate concentration of auxin and cytokinin PGRs favours rapid cell dedifferentiation, cell proliferation and results in the formation of callus (dedifferentiated mass of cells). The initiation of callus cultures is very important in plant biotechnology, since from callus shoots, roots and somatic embryos can be formed by manipulating the auxin and cytokinin ratio (Jain *et al.*, 2000, Brand and Ruan, 2000 and Devi *et al.*, 2004).

The PGR and naturally occurring hormone, ethylene, also has a synthetic alternative often referred to as Ethophon and examples of its use are: (a) acceleration of the ripening process and facilitation of harvesting in some species of pepper, (b) breaking seed dormancy in an important medicinal plant, native to North America and (c) to increase latex flow in some species of rubber plants (respectively, see Mao and Motsenbocker, 2002, Maccia *et al.*, 2000 and Lopez *et al.*, 2000). Synthetic derivatives of natural PGRs are often chosen as they are cheaper and more stable than natural hormones. It is difficult to establish the exact effect that synthetic PGRs have on each type of culture as there are differences between species and different cultivars (Li *et al.*, 2002). Taking into account the endogenously programmed developmental pathways of plants is an important prerequisite in understanding *in vitro* responses. The following section will proceed with this framework in mind and link plant development with *in vitro* manipulation.

1.5 *IN VITRO* TISSUE CULTURE

In vitro culture plays a huge role in modern plant biotechnology and is commonly used in horticulture, forestry and crop improvement programmes. The ability to regenerate whole plants from single cells was first discovered at the turn of the century by Gottlieb Haberlant. However, it was not until the 1930's that the role of auxins and vitamins was discovered, which enabled the *in vitro* culture of several different species. By 1960, Murashige and Skoog discovered that the addition of sucrose, basic salts and plant growth regulators could dramatically improve *in vitro* plant manipulation, and this allowed the successful *in vitro* manipulation and micropropagation of several plant species (Murashige and Skoog, 1962). *In vitro* tissue culture can be used to rapidly produce plants that possess desirable attributes such as disease resistance, environmental tolerance, greater yields and quality, via genetic engineering. It is also used extensively as method of protecting rare and important species, via tissue culture banks or cyropreservation. *In vitro* tissue manipulation is a multi-step process including a preparative stage, culture initiation, micropropagation, root induction, a hardening period and finally transfer of the resulting plant to a natural environment (see Figure 1.3).

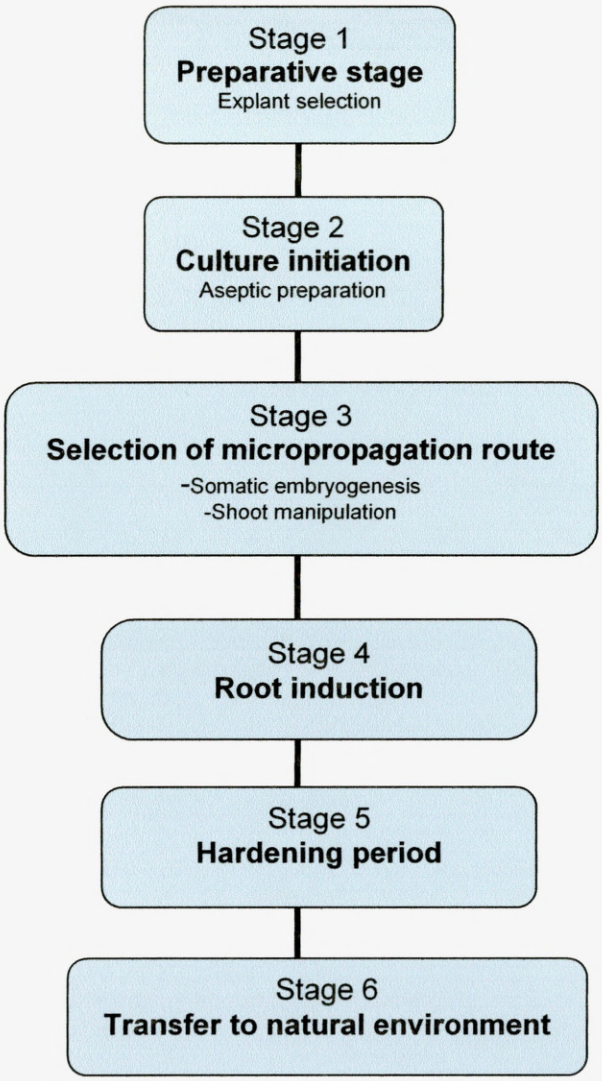


Figure 1.3: Overview of the four major steps involved in plant micropropagation.

In vitro tissue cultures are initiated from a sterile, carefully selected piece of the original plant. Explant selection is an extremely important stage in tissue culture as it can affect the quality of the regenerated plant. Wherever possible the explant should be selected from a plant that has been grown in a protected environment and the material itself should be selected from younger, rapidly growing areas of the plant, such as the apical shoot, auxiliary buds, young leaves or apical roots (Slater *et al.*, 2003). The selected explant should be easily sterilised, as this is essential to initiate

an aseptic culture, free from bacteria and fungi. Common sterilisation processes include washing the explant in 70% (v/v) ethanol for 30 seconds, cutting up the material aseptically and immersing the explant pieces into a 10% hypochlorite bleach solution for 5min. Finally, the explant material is washed clean by immersion in a series of sterile water flasks, until all traces of the bleach solution are removed; this procedure is often used for leaf material, though other modes of sterilisation include the use of active halogen (Stoimenov *et al.,* 2002) and heavy metal solutions (Slater *et al.*, 2003). There are several different types of tissue culture system, which have important roles in plant biotechnology, choice of culture method is often dependent on the nature of the material and its ultimate application in biotechnology programmes. There are two main types of plant culture, differentiated and dedifferentiated.

1.5.1 Differentiated cultures

Differentiated cultures comprise of organised material, such as shoot or root cultures and embryo cultures. Shoot and root cultures can be initiated by taking explants from the apical shoot and apical or lateral root tips. Shoot cultures form the basis of organogenesis culture methods; root growth in culture is generally unlimited and is widely used in genetic transformation.

1.5.2 Dedifferentiated cultures

Dedifferentiated cultures include callus and protoplast cultures, which are non-organised cells that proliferate in the presence of specific PGRs. Callus cultures and cell suspensions can be initiated by treating the explant with a combination of auxin

and cytokinin. Callus can be defined as a mass of growing, dedifferentiated cells and is commonly used to form somatic embryos and induce organogenesis in shoot and root cultures and is thus regarded as a very useful cell form in *in vitro* tissue culture (Lowe, 1996). Limitations of using callus is that it may, during long-term culture lose their embryogenic potential, and have reduced ability to form somatic embryos; however in most *in vitro* callus manipulations aged callus is not used. Moreover, dedifferentiation can predispose cultures to genetic change called somaclonal variation (Harding *et al.*, 1996). Habituation can occur in callus cultures, whereby cells no longer require plant growth regulators in the medium and cells continue to divide in an unorganised, chaotic fashion. Fully habituated cells are completely dedifferentiated and lose totipotency and the ability to form any organised structures (De Greef and Jacobs, 1979). Differentiated and dedifferentiated cultures are linked by a common process that is that callus is often used as the route to form embryos, through a process known as somatic embryogenesis. In contrast to shoot formation through organogenesis; alternatively embryo and shoot cultures can also be used to form callus cultures both processes are mediated by plant growth regulators.

Protoplast cultures, defined as a cell with the cell wall removed leaving behind what is commonly referred to as a "naked cell", can also be initiated (Davey *et al.*, 2005). The removal of the cell wall is a complex and delicate process and the resulting protoplasts are very fragile and prone to damage, which can cause the cell to lose viability (Slater *et al.*, 2003). Viable protoplasts are highly totipotent if the optimal production process is used for their formation and the correct chemical stimulus is applied. Protoplasts are capable of forming a new cell wall and undergoing mitosis, producing daughter cells, which can continue to develop in the normal way and form

fertile plants (Davey *et al.,* 2005). Protoplasts have been used to transfer useful agronomic traits via protoplast fusion and genetic engineering techniques. In brassica, traits such as enhanced resistance to blackleg (*Leptoshaeria maculariis*) (Hu *et al.*, 2002) and increased biomass (Qian *et al.*, 2003) have been introduced. In citrus fruits protoplast fusion has enhanced the resistance to citrus blight in several varieties (Costa *et al.*, 2003) and in eggplant varieties increased resistance to bacterial and fungal wilt has been achieved using protoplast fusion methods (Collonnier *et al.*, 2001). To summarise so far, the two main methods of plant regeneration are through somatic embryogenesis and organogenesis and each will be discussed in more detail in the following section.

1.5.3 Somatic embryogenesis

Somatic embryogenesis is the ability to produce morphologically and developmentally competent bipolar embryogenic structures derived from somatic cells. The ability of cells to undergo somatic embryogenesis was first discovered in carrot cultures in 1958 by Stewart and Reinert and this process is unique to the plant kingdom (Steward *et al.*, 1958 and Reinert, 1958). Since its discovery somatic embryogenesis has become a very useful method of plant regeneration and is also an excellent model for study of early plant regulatory and morphogenic events that take place during embryogenesis. Somatic embryogenesis can proceed via two pathways, direct somatic embryogenesis from differentiated cells or indirect somatic embryogenesis from dedifferentiated callus cells or cell suspensions, the most commonly used process is the indirect somatic embryogenesis method.

Somatic embryogenesis shares many of the stages of natural embryogenesis, but also differs significantly in others. Somatic embryos can be initiated from embryogenic somatic callus cultures and can be induced through the use of PGRs (often through removal of the auxin source, 2, 4-D for example, from the medium). The callus cells go through the normal stages, identified as globular, heart shaped and torpedo, and proceed to form cotyledons and plantlets, which can, after climate hardening and root formation, be used to regenerate a whole plant (see Figure 1.3). In natural zygotic embryogenesis the zygote, which is protected within a seed, also goes through the globular, heart shaped and torpedo stages. Once the cotyledon is formed this is the point at which the two processes differ as the zygotic embryo undergoes expansion, maturation, and desiccation and there is a dormancy period if required (species and environmental dependant). On the onset of germination the matured cotyledon breaks out of the protective seed casing and continues its normal growth pattern (see Figure 1.4).

Over the last twenty years there has been a dramatic increase in the number of species that have been successfully regenerated and improvements on existing micropropagation methods via somatic embryogenesis. Especially interesting are some very important crops, crucial in the developing world, such as *Ipomeoa batatas* or sweet potato (Al-Mazoorei *et al.*, 1997). Somatic embryogenesis was also found to be a crucial method of producing artificial seeds in sweet potato (Chee and Cantliffe, 1992).

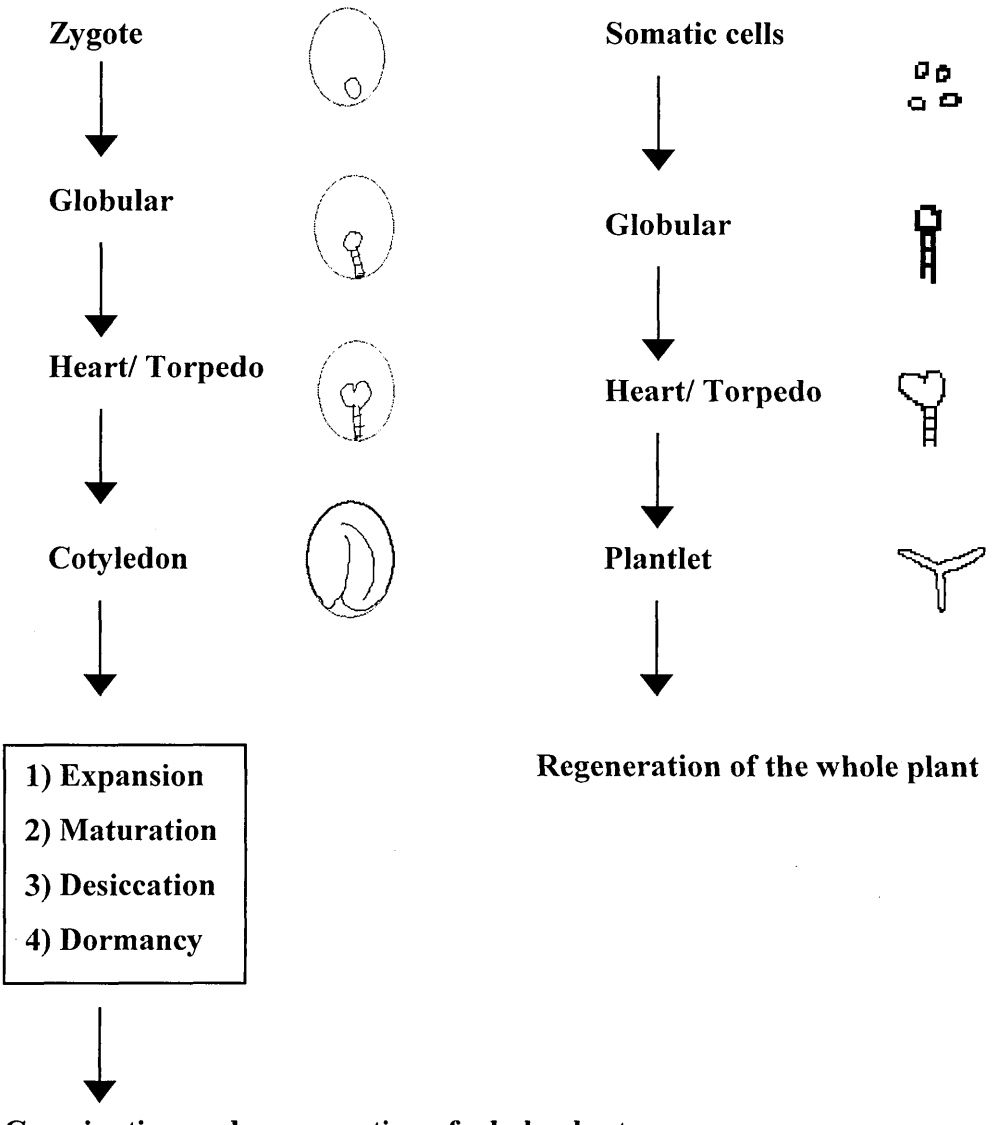


Figure 1.4: Figure describes the embryogenesis process that occurs in plant cells in the natural environment (left hand side) and somatic embryogenesis that occurs in somatic cells maintained in *in vitro* tissue culture in response to plant growth regulators (right hand side).

Somatic embryogenesis has been particularly utilised in the clonal forestry of conifers. The first plantlets from forest tree species were produced in the 1960's via adventitious shoots, however more recently the forestry industry has focussed its attention on micropropagation through somatic embryogenesis (Merkle and Dean, 2000). The production of somatic embryos has immediate potential advantages for

forest tree species, such as higher multiplication rates and huge potential for large scale ups, not to mention the easier delivery of species through artificial seed technology. However, the success of somatic embryogenesis in many woody species appears to have its limits, such as low frequency in the regeneration of desirable clones (Merkle and Dean, 2000). Recent research into these problems has been focussed on improving the quality of the somatic embryo through experimenting with the PGR and media components and in some species of pine, this led to a higher percentage of mature embryos being formed and thus increased regeneration rates (Li *et al.*, 1998). The actual changes made to the tissue culture conditions appear to be very genotype specific, which means that, potentially, regeneration rates of several species could be improved by finding exactly which conditions suit the genotype and this prospect has renewed the focus of research in pine species.

1.5.4 Organogenesis

The second method of micropropagation is via organogenesis. Organogenesis relies on the production of roots, shoots or leaves i.e. actual organs. There are three main methods of organogenesis; the first two rely on the formation of adventitious organs either directly from the explant or through an initiated callus. The third method of organogenesis can occur through auxiliary bud formation and growth; this will also result in the regeneration of the whole plant. The production of organs via organogenesis is stimulated by the application of PGRs in different combinations depending on which organ is required, bearing in mind that higher cytokinin induces shoot production and higher auxin concentration encourages root formation.

After the establishment of plantlets either via somatic embryogenesis or shoot multiplication via organogenesis, the next stage in micropropagation is to form roots on the plantlets. This is accomplished by transferring the plantlets onto rooting medium, which will contain a source of auxin to induce rooting. Root formation is essential if the plant is to survive in field conditions and then a period of plant "hardening" is required where the plants are transferred from the aseptic, controlled lab conditions to the greenhouse environment, for acclimatisation purposes. An overall schematic diagram of the four major steps involved in plant micropropagation is given in Figure 1.3). The advantages of micropropagation are that it allows rapid multiplication of superior disease-free clones and sexually derived sterile hybrids. It makes it easier to transport and store plants for large-scale projects and it also means that plants can be produced irrespective of the season. Micropropagation alone can be responsible for superior growth in plants and can result in plants with better foliage or more flowers.

1.5.5 In vitro recalcitrance

Recalcitrance is a problem that affects many species and, although relatively rare, it does present big problems in tissue culture. Recalcitrance can be defined as "the inability of plant cells, tissues and organs to respond in tissue culture" (Benson, 2000b). Current, research focussed on overcoming recalcitrance is based on gaining a better understanding of tissue culture responses and the improvement of tissue culture conditions. Since the Society for *In Vitro* Biology Congress hosted the Symposium entitled "Do We Understand *In Vitro* Plant Recalcitrance? (In Vitro Cellular &

Developmental Biology, Vol. 35, Part II, 1999) there have been several advances in species that have been previously recalcitrant to *in vitro* manipulation.

There are many different factors that may affect recalcitrance, such as choice of explant material, the time in the life cycle of the plant and the maturity of the explant selected. In *in vitro* cultivars of *Rosa* spp, Hsia *et al* (1996) showed that the distance of the explant from the apical meristem influenced the successful manipulation of the species in tissue culture. Several species of plants undergo seasonal changes during development and the timing of the explant selection in relation to the seasonal stage has also been shown to overcome recalcitrance in several species of temperate trees (Meier and Reuther, 1994). In some tropical species, which lack seasonal changes, the time of explant selection in relation to the plant life cycle was imperative to overcoming recalcitrance.

The maturity of the explant has been shown to be a factor in recalcitrance, in some cases the use of mature explants has been more successful than seedling derived explants (Parra and Amo-Marco, 1997). In other species the use of juvenile explant material was found to be more successful than mature explant material in overcoming recalcitrance (Miguel *et al.,* 1996), therefore it appears to be species dependant. In addition to explant selection there are also several physical factors, such as vessel type and size, temperature and light intensity to consider (Benson, 2000a). The optimisation of tissue culture components, (gelling agent, carbon source, strength and choice of PGRs and growth additives) can be beneficial in improving micropropagation performance.

Optimising the PGR concentrations in several recalcitrant species has been shown to improve cassava genotype response in *in vitro* tissue culture. Guohua, (1998) found that by carefully adjusting the PGRs used shoot organogenesis and somatic embryogenesis in this previously recalcitrant cassava species could be promoted and enhanced. The use of very potent bi-functional PGR thidiazuron which has cytokinin and auxin properties was shown to be successful in overcoming recalcitrance in woody species (Murthy, 1998) and in promoting somatic embryos in species of orchids (Huan *et al.*, 2004). However, the long term use of such potent auxins can be detrimental to the plants (Murthy, 1998). Ethylene has been shown to have both positive and negative effects on growth, in *Echinacea angustifolia* DC. The application of ethylene was shown to overcome the problems of seed dormancy in this species (Maccia *et al.*, 2000), however in chile peppers the reduction of ethylene, through the application of silver nitrate to the medium, enhanced shoot development and made regeneration capable through organogenesis (Hyde and Philips, 1996).

Changes to the media protocol can also help to overcome recalcitrance in certain species. Acacia are important commercial trees, which are abundant in Australia, America, India and Africa. They have particular value in reforestation and the reclamation of wasteland and are a very important source of firewood, tannins and flavourings. There has been a significant amount of research in this species to improve the quality of the regenerated plants where researchers have investigated the use of different types of explants, and their responses to PGRs. In many cases the response was species dependent. The significant improvements made to current routes of micropropagation have been reviewed by Vengadesan *et al.* (2002).

Recalcitrance can impede the development of biotechnological improvement strategies for crops of importance to the developing world. Within the context of this thesis, studies that assist a current understanding of *in vitro* recalcitrance provide an important justification for fundamental studies. Grain legumes are the main dietary protein sources for many people in developing countries such as Asia and Africa. Over the last few decades there have been great improvements on the previous methods of propagation in this extremely valuable crop (Gulatia and Jaiwal, 1992, Gulatia and Jaiwal, 1994). Gulatia *et al.* (1992) obtained successful regeneration through organogenesis and this is one of the earliest reports of successful somatic embryogenesis. In 1998 Amitha *et al.* (1998) reported regeneration via somatic embryogenesis in some species of *Vigna* and subsequent improved tissue culture conditions have allowed the micropropagation of the *Vigna radiata* L species to advance even further. Devi *et al.* (2004) have successfully produced viable plants from this species by determining the best type of PGR and its optimum concentration; in addition best results were achieved by including the additive IBA in the basal medium. They also increased the regeneration percentage by encapsulating produced embryos in synthetic seeds, allowing more protection against plant pathogens and physical factors.

Physical factors such as light and temperature effect the production of somatic embryos. In melons, a two week dark incubation during the initiation of embryos, followed by a 16/8 hr light/dark cycle during the developmental period was shown to be optimum for increasing the number of somatic embryos per explant (Guis *et al.*, 1997). Kevers *et al.* (1995) have shown that in habituated, stressed *B. vulgaris* cell lines, dark incubation promoted growth thought to be due to a decrease in oxidative

stress. To conclude at this stage, recalcitrance has huge implications for *in vitro* tissue as it is a major block for biotechnology programmes. There is however, to date, no understanding as to the fundamental basis of recalcitrance and to overcome the problem is an important area of *in vitro* research. Therefore, to set the scene for the rest of the thesis, the following sections will consider in more detail aspects of *in vitro* stress physiology that can lead to abnormal growth and development responses and including their epigenetic and genetic implications.

1.5.6 Hyperhydricity (vitrification) of shoot cultures

Vitrification or hyperdricity of shoot cultures is the process whereby regenerating shoots irreversibly swell and become physically distorted, often showing wrinkled surfaces and are often translucent and pale in colour. Vitrification was used for the appearance of these cultures as they appear "glassy". The abnormal "syndrome" often leads to necrosis of shoots and inevitable cell death; shoots that are undergoing vitrification are often described to be hyperhydric (Warren, 1991). It has been suggested that vitrification may be caused by water logging of the cultures as it is more common in cultures that are maintained in a liquid medium culture, however it also occurs in shoots that are maintained on solid medium. Certain types of carnation are very prone to hyperhydricity, in particular the *Dianthus caryophyllus* species (Saher *et al.*, 2005) and all species in the Carophylliceae family (Debergh *et al.*, 1992). One theory is that high humidity and accumulating ethylene levels, resulting as a consequence of tightly closed culture vessels is the cause of vitrification. There have been several tried and tested methods to reduce hyperhydricity in shoot cultures. Buddendorf-Joosten *et al.* (1996) investigated the effects of different gaseous systems

on potato plantlets and showed that by using a flow culture system they could control the humidity and gas mix. They found that decreasing the humidity in the vessels did not significantly affect growth, but they did find that be decreasing the O_2 concentration and by increasing the CO_2 concentration there was a significant increase in leaf size and freshweight. Buddendorf-Joosten *et al.* (1996) also confirmed that increased ethylene levels inhibited shoot growth.

The use of an ethylene absorbent has been used in several species to determine whether this will reduce hyperhydricity and generally improve growth rates in shoot cultures. Some success using activated charcoal in the media to remove ethylene has been observed (Mensuali-Sodi *et al.*, 1993) and more recently the use of an "ethylene scrubber", which utilises the capacity of $KMnO_4$ to absorb ethylene has been described (Park *et al.,* 2004). The latter workers compared a completely sealed vessel, a gas permeable vessel and a completely sealed vessel fitted with an ethylene scrubber. The gas permeable vessel produced normal shoots and very little ethylene was detected in this vessel, suggesting that growth was improved using this type of configuration. It was observed that the completely sealed vessel that contained the ethylene scrubber also produced normal shoots, without any signs of vitrification, suggesting that the removal of ethylene in a completely sealed vessel was sufficient to prevent vitrification. No ethylene was detected in the sealed vessel containing the ethylene scrubber, confirming that ethylene appeared to be directly linked to vitrification in this species. Studies to overcome hyperhydricity are ongoing and with improving propagation methods and media components for many species it may be overcome.

1.5.7 Genetic instability

In vitro tissue culture of plants is also known to cause somaclonal variation, a process that leads to regenerated plants having different phenotypes to the original plant. Somaclonal variation is a heritable change in plants regenerated from tissue culture and was first defined in 1981 by Scowcroft and Larkin (Scowcroft and Larkin, 1982) as "*genetic variation occurring in plants regenerated from cultured cells or tissues*". Contemporary definitions of somaclonal variation divide the phenomena into two types: genetic variation and epigenetic variation and changes can include activation of transposons, DNA amplification, point mutations and DNA methylation.

It was first recognised that somaclonal variation could exploit the potential of tissue culture to induce variation which could be exploited by tissue culturists to introduce advantageous traits to valuable crops, such as sugarcane (resistance to Fiji disease virus) and potato (altered tuber morphology) (Stafford, 1991). To exploit a somaclonal variant in the long term it is important to consider the stability of the variation, i.e. is the acquired resistance maintained throughout subsequent generations? As in some cases the new trait can be lost over subsequent regenerations. Therefore extensive field studies are required to test that the tissue culture derived variations are stable in the long term.

Somaclonal variation is exploited in a wide range of species to introduce a desirable trait such as improved disease resistance, drought or salt resistance, cold resistance, or simply as a means of improving the yield or ornamental value of a species. The ICARDA (International Centre for Agricultural Research in the Dry Areas) are

currently working to try and produce safer varieties of the legume *Lathyrus sativus* (grass pea). This is a highly drought tolerant species, which are found in Bangladesh, Nepal and Ethiopia and during drought and famine this species may provide the only food source available in the poorest areas. However if this variety of legume is eaten in large quantities it is toxic due to a build up of the neurotoxin beta-odap, which is a natural zinc carrier in the plant and can lead to spastic paralysis of the legs, commonly referred to as lathyrism. Thus the ICARDA are attempting to produce stable lines of *Lathyrus sativas* with lower levels of beta-odap, via somaclonal variation (Baum, 2005).

However, in the context of this thesis, somaclonal variation can also prove disadvantageous as unwanted traits can be produced and the process involves the production of plants and cultures that are stressed and/or deleteriously impacted by genetic change. This has longer-term implications as in breeding, particularly in industry, if an un-wanted variety is produced, which, for example, may result in a decrease in a particular pigment or reduction in an important chemical in the plant. This can lead to lower yields from each plant and may irreversibly affect the viability of the process due to increased costs. The critical variables that are involved in somaclonal variation are dependant on the genotype in question, the source and type of explant used, the duration of the culture, and the culture conditions such as light and temperature are all possible contributors. As *in vitro* ageing can have the potential to affect somaclonal variation (Scowcroft, 1984), the implications of ageing in bringing about deleterious genetic change require some further consideration. This may have particular impacts for those *in vitro* programmes that use genetic transformation.

1.5.8 Transformation

Transformation is considered in this thesis with respect to two issues: (1) recalcitrance (see above) can impede the regeneration of transformed cultures and (2) phytopathological transformation can lead to the development of neoplasia in plants *in vivo*. Transformation in *in vivo* plants systems is mediated through infections involving *Agrobacterium tumefaciens* species, the bacterium inserts its T-DNA into the plant cell, which causes plant cell to proliferate uncontrollably producing tumours that form on the surface on the plant. The use of transformation in *in vitro* plant systems is a very useful tool and is widely used in many current biotechnology programmes.

The advent of recombinant DNA in the 1970's allowed the genetic manipulation of plants to enter a new stage. Genes and traits that were previously unavailable through traditional breeding methods became available through DNA recombination. DNA recombination allows genes from sexually incompatible plants, animals or bacteria to be introduced to give the desired trait. It involves transfer of the desired genes into the plant cell genome, then regeneration of the whole plant via one of the micropropagation methods discussed previously. There are several different types of genetic transformation and success is very dependent on genotype. Methods of transformation include, the *Agrobacterium*-mediated transfer, direct gene transfer, polyethylene glycol (PEG) mediated gene transfer, electroporation, microinjection and microprojectile bombardment (Mohan Babu *et al.*, 2004). Currently, the most widely used method for transferring genes into plants is *Agrobacterium*-mediated transformation.

Agrobacterium is a naturally occurring pathogenic soil dwelling bacteria that has the ability to transfer its DNA into the genome of a plant and cause tumours. When a plant is wounded it emits certain chemical signals, which appear to activate the *vir* genes of *A. tumefaciens* and lead to series of events which result in the transfer of the T-DNA from the Ti plasmid to the plant's chromosome (see Figure 1.5). *Agrobacterium* infection and gene transfer occurs at the site of a wound in the plant, and causes a characteristic growth, known as a crown gall tumour (Fosket, 1994). The T-DNA enters the plant cell via the wound site and the bacterial DNA moves from the plant cytoplasm to the nucleus. It is not exactly clear how exactly this process occurs, nor how the DNA becomes integrated into the genome of the plant and the T-DNA genes are expressed. One speculation is that the T-DNA waits until the plant DNA is being replicated or transcribed then inserts itself when the DNA is exposed (Fosket, 1994).

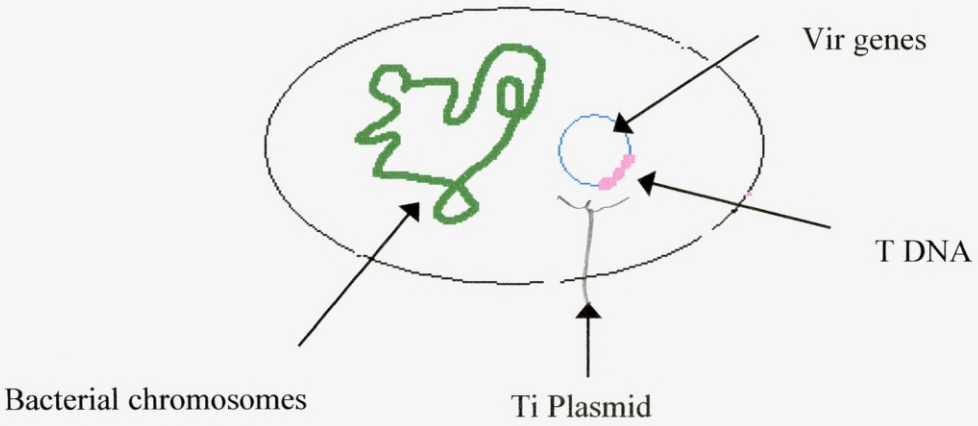


Figure 1.5: A cell of *Agrobacterium tumefaciens*. The DNA in an *A. tumefaciens* cell is contained in the bacterial chromosome as well as in another structure known as the Ti (tumour-inducing) plasmid. The Ti plasmid contains a stretch of DNA termed T-DNA (~20 kb in length), which along with a series of virulence genes that direct the infection process are transferred to the plant during the infection (Diagram adapted from the http://www.colostate.edu/programs/lifesciences/TransgenicCrops.html).

Advantage has been taken of this naturally occurring transfer mechanism and DNA vectors from the tumour-inducing plasmid DNA found in the *Agrobacterium* bacteria

have been designed to carry desired genes into the plant; this method is frequently used to produce herbicide, pesticide and chilling tolerance in economically important species. To harness this bacterium as a transgene vector, the tumour inducing section of the T-DNA must be disabled, while retaining the T-DNA border regions and the *vir* genes. The transgene must be inserted between the T-DNA border regions for successful transfer into the plant (Wong, 1997).

The *Agrobacterium* method is very successful in dicot plants such as soybean and tomato species and has more recently been successfully applied to maize, wheat and certain rice species. In tree species the *Agrobacterium*-mediated transformation system was the method of choice for angiosperms, however in gymnosperm trees transformation was achieved via microprojectile bombardment, as certain gymnosperm species, in particular conifers, were recalcitrant towards the standard *Agrobacterium* strains (Peña and Séguin, 2001). Antibiotic and herbicide resistant transgenes were successfully inserted into gymnosperms via the microprojectile method, however the percentage of untransformed cells was very high and the lack of a good reporter gene made the elimination of the untransformed cells difficult (Peña and Séguin, 2001). The development of a highly fluorescent protein (β-glucuronidene) that could be used as a reporter gene made the elimination of untransformed tree cells much easier (Tian, 1999) and may have increased the efficiency of the microprojectile method.

Genetic transformation is considered an invaluable tool in plant biotechnology programmes and has produced some very important and much improved crop species. However, *in vitro* recalcitrance and the deleterious genetic changes often associated

with it can have enormous impacts in the ability to produce stably transformed cultures and plants. Recalcitrance can impact the ability to maintain cultures in a totipotent state that allows post-genetic manipulation regeneration. Transformation in some species, however, can lead to genetic instability, which may predispose cultures to recalcitrance and habituation, thus decreasing responsiveness and impairing biotechnology programmes. Advances in genetic transformation and the increased understanding of the mechanisms surrounding the *Agrobacterium*-mediated method and, particularly, the role of the *vir* genes in the T-plasmid (Hansen and Wright, 1999) has allowed this method to be successfully applied to previously unresponsive species of gymnosperm trees, such as pine and spruce (Wenck *et al.*, 1999). One major advance in tree transformation is the successful insertion of a transgene that decreases the lengthy regeneration period in trees by reducing the juvenile phase, which is the main drawback in tree transformation (Weigel and Nilsson, 1995). This allows the analysis of the mature traits to take place sooner, speeding up the entire process, thus increasing efficiency.

Transformation in certain rice varieties, in particular japonica and indica has proved difficult in the past. During the initial years protoplast and electroporation were the only systems available as these species were unresponsive to *Agrobacterium*-mediated transfer though Shimamoto (1989) and Datto (1990) did manage to produce fertile transformed rice plants via these methods and since then these techniques have been used frequently (Christou *et al.*, 1991) but both methods have drawbacks. Regeneration from protoplasts is very time consuming, laborious and both methods are very genotype dependent. These methods are also associated with frequent and problematic somaclonal variations, leading to rice plants that take longer to flower

and have reduced fertility (Lynch, 1995). Another common problem is these methods can result in multiple copies of the transgene being inserted into the host genome (Rao *et al.*, 1995). In 1994 it was reported that the use of super binary vectors and the *Agrobacterium*-mediated transfer method were successful in japonica varieties (Hiei *et al.*, 1994). This procedure has now been successfully applied to elite varieties of indica rice to give resistance against yellow stem borer and sap sucking insects (Ramesh *et al.*, 2004). The *Agrobacterium* method provides a simple and very effective single DNA copy integration facilitated by the T-DNA transfer and thus eliminates the integration of multiple genes.

Somaclonal variation is thought to be involved in unsuccessful transformation in a number of species (Bhat and Srinivasan, 2002). For example, sorghum is one of the most recalcitrant cereal species to genetic transformation and many of the transgenes were found to be silenced after transformation. Emani *et al.* (2002) investigated the transgene silencing in this species and discovered that if the transformed cells were treated with 5-azacytidine (aza-C) the inserted transgenes could be reactivated suggesting that DNA methylation plays a role in hampering the transformation process in sorghum. In cucumber transgenic plants produced fruits upon self-pollination, yet there was no seed development (Mendel, 1995). Several steps are being taken to attempt to decrease somaclonal variation during genetic transformation and the recent procedures that have been applied to *Arabidopsis* are being extended to other species, such as rice and peanut (Bhat and Srinivasan, 2002).

1.6 BACKGROUND TO *IN VITRO* TISSUE CULTURES USED DURING THIS STUDY

1.6.1 History of plant species

B. vulgaris (sugar beet)

Alexander the Great reported the cultivation of sugar cane in India as far back as 327 B.C. Sugar was primarily imported to Europe from Asia, where it was cultivated by China and India. The Ottoman Turks controlled the trade routes and they extracted large tolls from traders going to Asia, which made sugar very expensive and consequently European traders began to look for alternative routes to Asia. Christopher Columbus made his high-risk journey and failed to find Asia, however he did find the Americas and on his return introduced sugar production to Spain, the Canary Islands and Santo Domingo. The geographical spread of sugar produced from sugar cane lead to improved yields, lower production costs and greater availability, which in turn decreased the cost of sugar dramatically, thus losing its identity as a luxury item (Inkson, 2005).

The production of sugar from sugar cane in many parts of Europe was not possible due to the poor climate but matters changed somewhat when the presence of sugar in beets was discovered in 1747. Initially, the yields of sugar were very low but after much research improved procedures became economically feasible. The first sugar beet factory was founded in Kunern, Germany in 1802 and the production of sugar from beets flourished in Germany and France. The UK did not take the opportunity to manufacture sugar from beets on their own soil as they had a ready supply of sugar

produced from sugar cane refineries in British colonies in the West Indies and also imported beet sugar from France and Germany. During the First World War the levels of sugar being imported into the UK dropped dramatically and it was during this time that the British sugar beet industry was founded. Sugar produced from sugar beet has thrived in the UK since then and today it is estimated that the UK alone produces 9 million tonnes of sugar annually.

G. max (soya bean)

The soya bean is one of the oldest vegetables known to man; it was first cultivated in China prior to 3000 BC and has been consumed for over five thousand years. Soya bean is native to the Far East and China and was introduced to the United States in the 19th Century. Mass production of soya in the USA began during the 2nd World War when crops in China were destroyed. Soya is now the second largest and most valuable crop in the USA (after corn) and approximately two thirds of food products contain some form of soya derivative (Reynolds, 2005) and this percentage will probably continue to increase with the consumer demand for vegetarian and healthier alternatives (Debruyne, 2002).

Before soya can be used in foodstuffs the beans must be cleansed, cracked, dehulled and rolled into flakes, which ruptures the cell for easier extraction. Soya bean oil is extracted and refined in a variety of methods and then used directly in food products, whilst the material that is left over is used in livestock feed. The soya bean protein content is comparable to the quality of protein found in eggs and meat. Soya oil is

also very low in saturated fats and has no cholesterol, therefore is a healthier alternative to other commonly used oils.

1.6.2 Origin of cultures used during this study

B. vulgaris

The *B. vulgaris* cultures were originally initiated by De Greef *et al.* (1979). *B. vulgaris* (strain F3S52) plants were grown on vermiculite with a balanced mineral salts solution for 4 weeks in a greenhouse with a 16/8hr day/night cycle. The leaves from these plants were selected as explant material and soaked in a solution of saturated calcium hypochlorite solution, acidified using acetic acid and then cut into small uniform pieces ready for callus induction. Leaf material was placed onto the callus induction (PGR supplemented) medium (see Appendix 6.1) and incubated in the dark for 5 weeks at 24 °C on solid medium in Petri dishes and after 5 weeks callus was formed. To determine exactly what culture conditions gave rise to shoot formation from leaf derived callus, cold treatments (varying in length and under continuous illumination) and different PGR combinations (200 different treatment combinations) were applied to the primary callus (see Table 1.2).

Incubation (weeks)		PGR combination (mg/L)	PGR combination (mg/L)
5	Callus induction	2,4-D (0.1)+ BAP(0.1)	IAA (1.0) + Kn or BAP (0.1)
0-3-6-9	Cold treatment	Kn or BAP (0.1-1.0) + GA_3 (0.1-1.0)	Kn or BAP (1.0) + GA_3 (0-0.2)
8	Post-vernalisation	Kn or BAP (1.0)	Kn or BAP (0-1.0) + GA_3 (0-0.2)

Table 1.2: Combination of plant growth regulators and cold treatments applied to *B. vulgaris* callus cultures as a means of determining which culture conditions gave rise to shoot production from an original leaf derived callus (De Greef and Jacobs, 1979).

A cell line which was capable of producing shoots was isolated from the following treatment conditions: callus initiated on plant growth medium supplemented with plant growth regulators (PGRs) IAA (1.0mg/L) + Kn (0.1mg/L) over five weeks, followed by cold treatment on plant growth medium containing Kn (1.0mg/L) + GA_3 (0.2mg/L) over a nine week period and finally regenerated on plant growth medium containing Kn (1.0mg/L) + GA_3 (0.2mg/L). After eight weeks the formation of a "chlorophyll-less structure", which resembled a petiole, was observed. At the base of this structure a dark green bud developed which was removed and placed on PGR free medium where it was subcultured every 4 weeks. After 2 subcultures (8 weeks) a normal plantlet was formed. A loose greyish coloured friable material formed at the base of the plantlets, which was separated easily from the plantlet and placed on PGR free medium. After one subculture (4 weeks) the callus turned green then went on to form abnormal shoots and very occasionally normal plantlets.

These abnormal shoot cultures have been maintained in the same tissue culture conditions and are the habituated organogenic shoots cell line (HO). Studies over the years on this cell line have observed several abnormalities in this cell line, thought to

be a result of a progressive loss in totipotency and is regarded as a neoplastic progression (Gaspar, 1995). The original callus cell line always contained small amounts of very white callus and to determine whether this apparently dedifferentiated callus was capable of growing in the total absence of any external PGR supply (and completely independent from the differentiated cells) it was isolated and transferred to PGR free medium. It was found that this white callus was capable of still producing abnormal leaves but after 3 subsequent subcultures where only the white dedifferentiated material was transferred the ability to form leaves was lost. Thus a true dedifferentiated callus capable of proliferating in the absence of PGRs was produced. These cell lines discussed above are the cell lines N1 and N3 used during this study. A normal non-habituated (PGR dependent) dedifferentiated *B. vulgaris* callus (N) taken from the same mother plant was used as a comparison cell line. This unique set of *B. vulgaris* cultures was very kindly donated in 2001 for use during this study of habituation by Professor Thomas Gaspar from the University of Liege in Belgium, who is the leading researcher in the field of plant habituation. To facilitate and introduce these cultures their morphologies are shown in Figure 1.6.

The *B. vulgaris* cultures provide an excellent set of cell lines for the study of habituation, as each is considered to be at a different stage of habituation. Cell line N is not habituated and resembles a "normal" chlorophyll producing callus (see Figure 1.6, **N**), which is still dependent on plant growth regulators to promote continuous dedifferentiated growth. Cell line HO is a habituated cell line, which is still organogenic and produces shoots in the absence of plant growth regulators (see Figure 1.6, **HO**). Cell lines N1 and N3 are both fully habituated cell lines that are non-organogenic, not capable of chlorophyll expression and are able to sustain

dedifferentiated growth in the absence of plant growth regulators (see Figure 1.6, **N1** and **N3**). These cell lines (N1 and N3) are considered to have lost all totipotency and have been previously compared to mammalian cancer cells (Gaspar, 1999a). Due to the many similarities between these fully habituated cell lines and mammalian cancerous cell lines they can be considered as true plant cancer cells. For an overall summary of the general characteristics of *B. vulgaris* cell lines see Table 1.3.

In a normal (PGR dependant) callus growth generally occurs through meristems and the only differentiation that takes place is the formation of xylem cells (Crèvecoeur *et al.*, 1987). Callus growth (PGR regulated) however does resemble that of a tumour thus is often regarded as a primitive "neoplastic growth". The habituated organogenic cell lines are thought to be undertaking neoplastic progressions which have led to the abnormalities in the cells lines, as a result of malfunctioning meristems, organogenic tumours are not thought to be as progressed as non-organogenic cancerous cells (fully habituated cell lines). The fully habituated cell lines N1 and N3 have lost all totipotency and have completely un-functional meristems and are true cancerous plant cells. This unique set of *B. vulgaris* cultures will be used to determine the effects of habituation and neoplastic progression on the morphological and antioxidant properties of the cell.

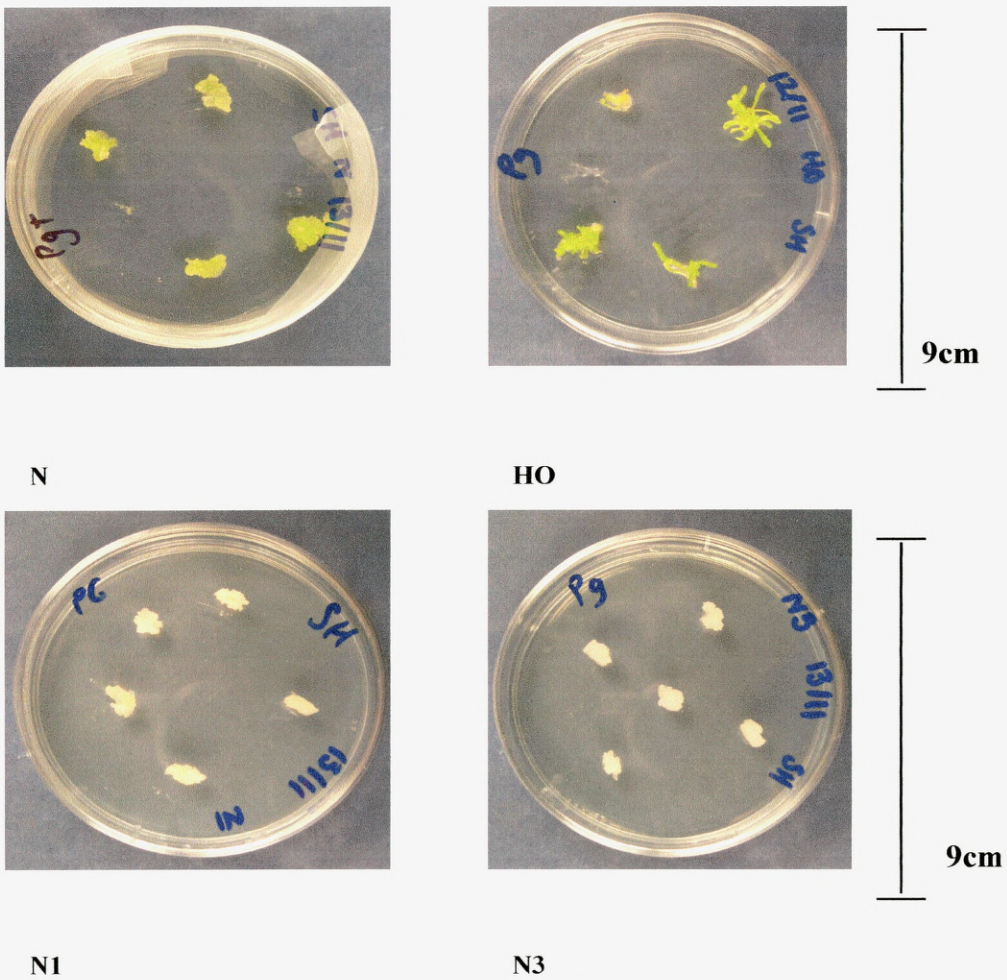


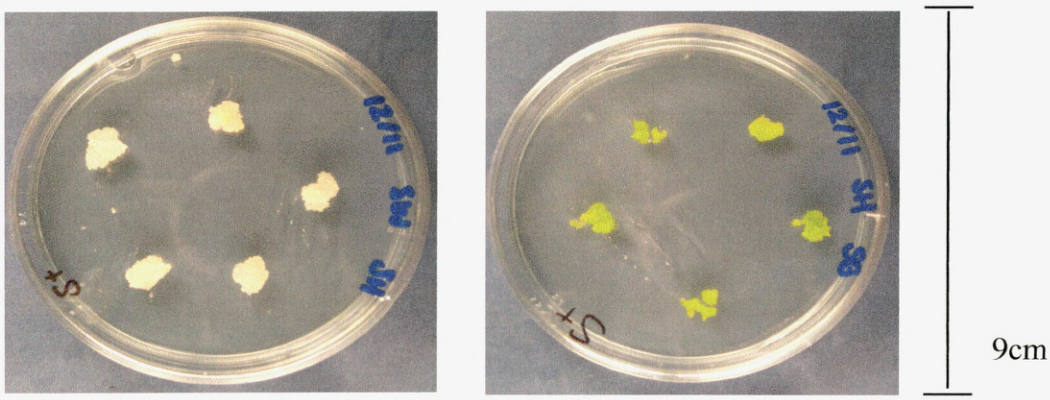
Figure 1.6: *B. vulgaris* cell lines. N= Non-habituated PGR dependent normal callus, grown on PG° medium supplemented with PGRs, BAP and 2,4-D (see Appendix 1) photographed 7 days through the subculture cycle; HO= Habituated organogenic, shoot producing material, grown on PGR free PG° medium photographed 7 days through the subculture period. N1 + N3= Fully habituated, non-organogenic callus grown on PGR free PG° medium, photographed 7 days through the subculture period. Scales are relative to a 9 cm Petri dish.

Cell Line	Type	Colour / pigment	Habituated
N	Callus, dedifferentiated	Green (pigmented)	No
HO	Organogenic / Shoots	Green (pigmented)	Yes
N1	Callus, dedifferentiated	White (non-pigmented)	Yes
N3	Callus, dedifferentiated	White (non-pigmented)	Yes

Table 1.3: Table showing a summary of form, colour and habituation status of *B. vulgaris* cell lines

G. max

G. max cultures were obtained from the John Innes Centre, Norwich approximately fifteen years ago (from 2004) and have been serially subcultured at the University of Abertay Dundee since then. There are two *G. max* cell lines denoted SW and SG. SW is a white (non-pigmented) dedifferentiated callus maintained on medium supplemented with the plant growth regulators 2, 4-D and NAA (see Figure 1.7); this cell line has lost the ability to produce any chlorophyll pigmentation. The green (SG) cell line is a very similar callus, grown on the same medium as the white (SW) cell line; however it is still capable of producing chlorophyll pigmentation (see Figure 1.7). These cell lines are not habituated as they still require plant growth regulators to grow in a dedifferentiated state however they are considered to be significantly "aged" cultures. For a summary of form, colour and age see Table 1.4. These two cell lines will be used to study the effects of *in vitro* ageing on the morphological characteristics and antioxidant potential.



SW **SG**

Figure 1.7: *G. max* cell lines. SW= Soya white and SG= Soya Green; both are aged PGR dependent callus grown on basal medium containing PGRs 2,4-D and NAA (see Appendix 1) photographed 7 days into the subculture period. Scales are relative to a 9 cm Petri dish.

Cell line	Type	Colour	Age (Years)
SW	Callus (dedifferentiated)	White (non-pigmented)	15
SG	Callus (dedifferentiated)	Green (pigmented)	15

Table 1.4: Table to show form, colour and age of *G. max* cell lines

1.7 INTRODUCTION TO PLANT CANCER

In recent years, the subject of uncontrolled growth (cancer) has been the focus of considerable attention as a result of its life threatening nature in humans. The extensive study of cancer has unlocked information and provided comprehensive facts surrounding undifferentiated tissue growth in mammals, however understanding the complete biochemical basis of cancer remains elusive. In contrast, plant cancer is a relatively new area of research and until recently very little was understood about the biochemical basis of plant cancers. Cells in any organism are normally under tight developmental control and participate in a series of events including differentiation, proliferation, migration and cell death; all are tightly controlled in a highly organised fashion to give rise to a functional organism (Voet and Voet, 1995). In plant cells developmental processes are very much influenced by the meristem regions, which direct differentiation and proliferation and are responsible for forming a functional plant structure. When plants lose this developmental control through meristem dysfunction then dedifferentiated cells can arise to produce dedifferentiated masses.

Cancerous cells in mammalian cells exist in two different types: benign and malignant. Benign tumours include growth formations such as warts and moles,

which grow in a simple expansive manner and often remain encapsulated by a layer of connective tissue, which prevents the tumour invading neighbouring cells. Malignant tumours grow in an aggressive and invasive manner and move rapidly from cell to cell as they are inadequately limited and invade neighbouring cells to gain what they need to survive and grow. Benign tumours are rarely ever fatal; however malignant tumours can invariably be life threatening due to the rate they spread around the organism by invading vital tissues. There are two types of genes involved in cancer and its prevention: oncogenes and tumour suppression genes. Activation of oncogenes causes a rapid increase in cell division leading to formation of a tumour. Tumour suppression genes attempt to inhibit the action of the oncogenes.

Plant cells have the unique ability to organise themselves into organogenic or regenerating meristems and they can incorporate extra cells, which would possibly lead to malignant tumours in mammalian systems into normal regenerating meristems (Gaspar, 1999a). Due to this unique ability, malignant tumour formation is impossible in plant cells and consequently the only type of tumours observed to date in plants are the equivalent of benign tumours in mammalian organisms. Plant tumours caused by external pathogens, such as viruses and bacteria, are normally localised and appear at the surface of the plant at the site of infection.

There are four different pathological diseases in plants that are well documented to induce cancerous cells. Crown Gall disease and Black's wounds tumour diseases induce tumours in plants, via the action of external pathogens (Gaspar, 1999). Kostoff's genetic tumours are caused by a chromosomal imbalance in hybrid plants, which often results in the formation of cancerous cells and, finally, habituation a

process which occurs in *in vitro* plants cultures and leads to cells losing totipotency and which will lead to teratoma masses or where full totipotency is lost where they become true cancerous plant cells.

Crown Gall disease

In general, many strains of *Agrobacterium* are harmless to plants; however one particular strain *Agrobacterium tumefaciens* causes one of the most common of type of phytopathological tumours found in plants (see section 1.5.8). *Agrobacterium tumefaciens* is thought to affect over six hundred species of plants and is the most documented of the four cancer causing diseases. Crown Gall mainly affects dicotyledonous species, such as woody and herbaceous plants and can be identified by the appearance of galls of varying shape and size, normally on the lower stem and the roots. Crown gall disease is known to affect some very important commercial crops, including rice, sugar beet and grape (Escobar and Dandekar, 2003).

Agrobacterium tumefaciens and its related species *A. rhizogenes* and *A. vitris* invade plants by transferring their T-DNA from the TI plasmid into the genome of the plant (Tzfira and Citovsky, 2000). The T-DNA transferred from the *Agrobacterium* contains genes, which encode proteins that are involved in the biosynthesis of PGRs; these genes in the T-DNA are called the oncogenes. Activation of these oncogenes results in tumour formation (see section 1.5.8). Expression of additional T-DNA genes as well as the oncogenes causes the tumour cells to produce and secrete unusual carbon compounds called opines, which include octopine, nopaline, agropine and agrocinopine. The type of opine produced is not dependent on the plant but by the

inciting bacteria which is able to utilise the opine as a source of energy. It is common for *Agrobacterium tumefaciens* to be classified according to which opine they produce.

Agrobacterium tumefaciens enters the plant via a wound on the surface; it is thought that the bacterial cell can detect phenolics produced by the wounded plant. It is proposed that the bacterial cell uses a two component regulatory system involving V*ir* A and V*ir* G proteins. *Vir* A acts as the membrane sensor that detects and interacts with the phenolic signal and then *Vir* G regulates the cytoplasmic response, which induces all the other *Vir* genes required for attachment and transfer of T-DNA (Tzfira and Citovsky, 2000). The attachment occurs in a two-step process, the initial weak adhesion is followed by the synthesis of cellulose fibrils that anchor the bacteria firmly to the plant surface. Three specific bacterial genes have been identified to play a role in the attachment process (*chvA*, *chvB* and *pscA*) and the expression of all three appears to be crucial as mutants missing one or more of these genes appear incapable of attachment (Tzfira and Citovsky, 2000).

After attachment, the next step is the transfer of the T-DNA from the bacterium into the plant cell. The exact mechanism of the T-DNA transfer has not been completely established; however recent research using Arabidopsis is beginning to shed some light on the process (Qin *et al.*, 2003). It has been suggested that T-DNA complex passes into the plant cell nucleus by active nuclear uptake because the T-DNA complex (12.6nm) is too large to fit through natural nuclear pores (9nm). During active nuclear uptake the pores can expand to up to 23nm, which is sufficient for the T-DNA to gain access to the plant genome (Tzfira and Citovsky, 2000). The final

stage in the transformation process, which is still an area under significant research, is the integration of T-DNA into the genome. Unlike many other genetic elements, such as retroviruses, T-DNA does not encode functions for transport and integration, therefore this DNA sequence is non-specific. It is this property that makes the T-DNA very useful as any desired DNA sequence inserted between the T-DNA borders can be transferred into the plant genome, which makes T-DNA a very attractive tool for plant transformation (Tinland, 1996).

The discovery of *Agrobacterium*-mediated transformation was of vast importance for genetic transformation (Chilton, 1977) as it is more successful and more accurate than many direct gene transfer methods in many species. This method has been successfully used in a number of agricultural plants (Babu *et al.,* 2003) to increase disease resistance (Ramesh et al., 2004), drought resistance and to improve yields (Wang and Yaxin, 2005, Arawal *et al.,* 2004) and other ornamental novel characteristics such as flower symmetry or colour (Cui and Ezura, 2003). Before the *Agrobacterium*-mediated gene transfer can be carried out it is necessary to delete the gene, known as the phyto-oncogene that causes the uncontrolled cell proliferation and resulting tumour (gall). Once the tumour producing part of the T-DNA is disarmed the single or multiple genes, which code for the desired traits are inserted between the borders into the T-DNA. It is also necessary to add a gene that will make transformed cells identifiable; a commonly used method is the addition of an antibiotic resistance gene, which allows transformed cells to be selected from medium that contains that antibiotic (Fosket, 1994).

Wound tumour disease

Plant retroviruses are viruses that infect the plant and also the host insect; they can be split into three main genera, *Phytoretroviruses*, *Fijiretroviruses* and *Oryzaviruses*. *Phytoretroviruses* include wound tumour disease (WTV), rice dwarf virus (RDV) and rice gall dwarf virus (RGDF). Wound tumour disease (WTV) is the only retrovirus that affects dicots (Noda and Nakashima, 1995); this virus is a non-enveloped double stranded RNA virus, which is transmitted to plants through insects known as leafhoppers. Plants that are susceptible to WTV include *Lycopersian esculentum* (tomato), *Dianthus barbatus* (Sweet William) and selected *Nicotiana* species (Brunt *et al.*, 1996).

When a reterovirus, such as WTV infects a plant cell its RNA genome is first converted to DNA by the viral encoded RNA-dependent DNA polymerase (reverse transcriptase). This DNA is then inserted into the plant genome via the virus, where it can be copied by the plant during normal cell division. Contained within the sequences of the inserted genome are powerful transcriptional promoter sequences called long terminal repeats (LTRs). The LTRs promote the transcription of the inserted viral DNA, which leads to the production of new viral particles. At some point in the viral integration process rearrangement of the viral genome occurs, resulting in a portion of the host genome becoming incorporated into the viral genome; this process is known as transduction. Occasionally, this transduction can lead to the virus acquiring a gene from the host cell that is involved in controlling growth. This alteration in the host gene and the increased transcription rate due to its association with the retroviral LTRs, results in unrestricted cellular proliferation

leading to the formation of tumours. The virus replicates actively in both the insect and the plant hosts. Plant viruses behave in a very similar manner to many other viruses and adhere to the same stages of attack, attachment, penetration, synthesis, assembly, maturation and release. The host plant becomes systematically infected from the point of irritation often found where lateral roots emerge (Gaspar, 1999a).

Non –phytopathological genetic tumours

Genetic tumours are neoplastic growths that arise spontaneously in large numbers in interspecific hybrid plants (Wang and Rhee, 2000) in the absence of any external pathogens, such as viruses, bacteria and fungus. Genetic tumours were first described by Kostoff in 1930 when they were discovered in hybrid species of *Nicotiana* (Kostoff, 1930) and consequently are often referred to as Kostoff's genetic tumours. Cells isolated from genetic tumours are capable of proliferation in the absence of any PGRs in the medium (Nandi and Palni, 1997). There has been significant data published on the morphological and physiological properties of these genetic tumours (Wang, 1998), however until relatively recently there has been little published on the molecular aspects of these types of tumours (Wang and Rhee, 2000). Wang *et al.* (2000) have focussed their research on the identification of cyclin genes that may be responsible for the deregulation of cell division, which leads to the subsequent tumour.

Cyclins are a family of proteins, whose concentrations rise and fall at specific times during the cell cycle and are thought to play a crucial role in the eukaryotic cell cycle machinery. Plant cyclins have been identified that have very similar structures to the

mammalian cyclins A, B and D (Renaudin *et al.*, 1997). By isolating genetic tumour cells Wang *et al.* (2001) found that certain cyclins were expressed to a higher extent, therefore the determination of the function of these cyclin genes may shed some light on the molecular mechanism of cell deregulation, which is associated with the formation of genetic tumours.

In vitro habituation and neoplastic progression

Habituation occurs, like genetic tumours, in the absence of any external pathogens, and effects plants that are maintained in *in vitro* tissue culture. It is caused be the cells forming independence from the normally required PGRs in the culture medium, resulting in uncontrolled cell proliferation and the loss of ability to form any organised structures; habituated cells normally always exist in callus form. The following section overviews the process of habituation, which, as a subject is still under considerable debate as to its underlying nature.

1.7.1 Habituation

Habituation is a condition that affects cells maintained in *in vitro* tissue culture. It was first discovered in carrot cultures in 1942 (Gautheret, 1942) and has been defined as:

> ***"The acquired and hereditary capacity for autonomous growth in the absence of exogenously supplied in the tissue culture medium"***
>
> *(Gaspar, 1999a).*

Habituation is a relatively rare occurrence that effects callus cultures and in some very rare cases shoot cultures (Gaspar, 2000). A source of auxin and cytokinin are normally required in the plant growth medium to initiate and maintain dedifferentiated growth of plant cells. When cells acquire the ability to dedifferentiate in the absence of cytokinin, auxin or both and the cells can proliferate on PGR free medium there are classed as being habituated. Cells that are habituated against both cytokinin and auxin are considered to be fully habituated.

Auxins and cytokinins are the regulators that govern the growth of the plant and are added to the tissue culture medium in specific concentrations depending on the desired growth effect. Auxins are involved in cellular elongation, differentiation in root growth and the development of vascular tissue. Cytokinins act in concert with auxins to promote rapid cell division. To sustain autonomous or dedifferentiated growth auxins and cytokinins are required at appropriate concentrations to maintain hormonal balance. The reason for the cells ability to grow in the absence of the plant growth regulators is not completely clear; however it is thought that the independence is gained through an altered metabolism or sensitivity to hormonal and cell signalling triggers.

One possibility is that a source of auxin and cytokinin is produced from within the cell autonomously (Meins, 1987), therefore removing the need for exogenously added PGRs. Others suggest that PGRs have been replaced by another compound (produced *in vitro*) that mimics or replaces the PGR action (Persinger and Town, 1991; Kevers *et al.*, 1981; Gaspar, 1999b). In particular it has been suggested that cytokinin could be replaced by an alternative metabolite (Gaspar, 1999). Dihydroconiferyl alcohol

glucosides (DCG's) have been found to accumulate in cytokinin stimulated tissues, DCG's are derivatives of the phenyl propanoid pathway and have been isolated in *Catharansus roseus* L. crown gall tumours (Teutonico *et al.*, 1991). DCG's have been previously recognised as having cell division promoting activity and have been shown to replace cytokinin to promote cell division in cytokinin requiring tissues (Binns *et al.*, 1987). It is proposed that DCG's accumulate in cytokinin requiring tissues and are able to replace cytokinin and stimulate cell division; however this metabolite cannot stimulate all the processes that the natural adenine-derived cytokinin can, such as shoot initiation (Teutonico *et al.*, 1991), hence why habituated tissues are normally dedifferentiated. Altered ethylene production has also been proposed as a means of altering sensitivity towards PGRs (Hagège *et al.*, 1994); and changes in gene expression may also allow cell division to occur independently of hormonal regulation (Campbell and Town, 1991), however, to date, the actual source or replacement PGRs or the cause of altered sensitivity has not been universally confirmed.

Habituation can occur spontaneously or gradually and in most cases habituation is thought to be reversible. However Gaspar *et al.*, (1999) have suggested that there may be several degrees of habituation, which can be described as steps of neoplastic progression eventually leading to the onset of cancer in the absence of an external pathogen. Neoplastic progressions during habituation include cell rejuvenation with deficient differentiation, loss of ability to organise meristemic centres and loss of totipotency (Gaspar, 1999). Once cells reach the end of neoplastic progressions they are completely dedifferentiated and have lost all totipotency and can be classed as true cancerous plant cells (Gaspar, 1999a). During the early stages of habituation cells can

often be reversed from habituation as they frequently maintain totipotency, however as the cells lose totipotency the chance of reversibility decreases.

Long term serial subculture of plant tissues may contribute towards habituation, however some plant cells lines can be subcultured for many years and suffer no visible changes and can maintain full totipotency and regenerate roots, shoots and somatic embryos in response to applied PGRs. In many cases, as the tissues are serially subcultured they begin to lose totipotency and organogenic potential decreases (Collin and Edwards, 1998). Why this happens is a subject of major debate in plant biotechnology. Tissue culture medium has been refined to produce a substrate that enhances cell proliferation, a necessary requirement for micropropagation for which fast growth and fast yields are essential considerations. Encouraging cells to proliferate may mean that the number of cells "selected" for organogenesis (the formation of organised structures) is reduced and this causes a loss of pre-determined genetic variation required to maintain full totipotency. Moreover, it is important to consider that epigenetic changes, such as DNA methylation and/or somaclonal variation may reduce tissue culture competence (Gaspar *et al.*, 2000). An alternative explanation for the loss of totipotency is the possibility that there is an "unidentified" compound or compounds of high endogenous concentrations, present in explants that promotes organogenesis. When the explant is placed onto culture medium this unspecified substance either diminishes, or there may be an "unidentified" compound that accumulates in the cells after transfer to culture media which reduces organogenesis (Gaspar *et al.*, 2000).

The four *B. vulgaris* cell lines used in the current research are at various stages of habituation and each cell line is thought to be undertaking neoplastic progressions towards fully habituated cancerous cells. Cell line N is a normal callus, which is dedifferentiated by including PGRs in the medium. This cell line was not thought to have entered neoplastic progression. However, within the later time frame of this thesis, a recent publication has suggested that cell line N may in fact be undergoing some cytological changes that were previously only observed in the fully habituated cell lines (Häsler *et al.*, 2003), suggesting that it may be beginning to enter neoplastic progression spontaneously. Cell line HO is more advanced than the normal cell line (N) and is no longer responsive to PGRs. This cell line is still capable of producing shoots in culture in the absence of any PGR, demonstrating that this cell line is organogenic, therefore has not yet reached the fully habituated state. Cell lines N1 and N3 are considered to have reached the end of neoplastic progression and have attained a point of no return and can be considered true cancerous cells.

1.8 CELLULAR AGEING

Ageing and cancer from a pathological and developmental perspective could in many respects be classed as two entirely separate phenomena. Cancer is a disease of overgrowth, too much vitality and cells that cannot stop dividing. Ageing, on the other hand is usually pre-determined and programmed senescence and a progression towards the point at which the growth, of cells is superseded by a "wearing out" of support, maintenance and repair systems. However ageing and cancer appear to be related to each other in many respects, cancer is very much a disease associated with ageing, 60% of newly diagnosed malignancies are found in people aged over 60 and

70% of all deaths occur in this same group. The strong pathological and disease progression links between cancer and ageing has lead scientists to believe that there may be a strong biochemical and molecular link between the two. The three main questions that surround ageing and cancer are as follows:

(1) Is cancer an unavoidable part of ageing?

(2) Is cancer a result of a disease associated with ageing, without it being directly linked?

(3) Is cancer linked to ageing perhaps on a molecular-DNA level caused by the ageing cells influence on cells?

One suggestion is that cancer takes time to develop and this is why it occurs more frequently in aged systems. Do cells become more susceptible to cancer causing agents as they age or do the aged cell repair systems lose the ability to scan, locate and repair aberrant malignant cells?

Understanding what causes ageing, the phenomenon that occurs towards the final stages of life is one of the greatest challenges facing scientists to date. The term senescence can be defined as advancing age and the complex of ageing processes that eventually lead to cell death. Ageing is associated with a gradual slowing down in metabolic processes resulting in a decreased rate in the maintenance and repair processes. This leads to an accumulation in metabolic errors and increases in genetic damage. There are a number of different theories that surround ageing, a number of which are genetic based and several others which are non-genetic based i.e. involve non-genetic processes, such as free radicals, which often lead to genetic damage as the end result. Much of the research conducted on ageing has been carried out on

mammalian models as they tend to have a very predictable life span that is species dependent, which makes studying ageing effects much easier than in plants, which have very varied life spans and can have unusual growth patterns. Recently however there is an increasing amount of data being published on plant senescence and ageing (Yoshida, 2003, Quirino *et al.,* 2001, Noh and Amasino, 1999, Masclaux *et al.,* 2000).

In 1961 Hayflick *et al.* proposed that organism death as a result of ageing is caused by worn out tissues that cannot forever be renewed. They (Hayflick *et al.,* 1961) conducted a series of experiments to determine whether cells could divide an infinite number of times in cell culture. Their results showed that a population of normal human fibroblasts divided a finite number of times, after which the cells stopped dividing and entered what Hayflick termed the phase III phenomenon, where cell replication diminished and ultimately growth stops; this observation was termed the Hayflick limit (Hayflick and Moorhead, 1961) and they (Hayflick *et al.,* 1961) and other researchers (Morin, 1989) proposed that cell replication was limited by telomere shortening.

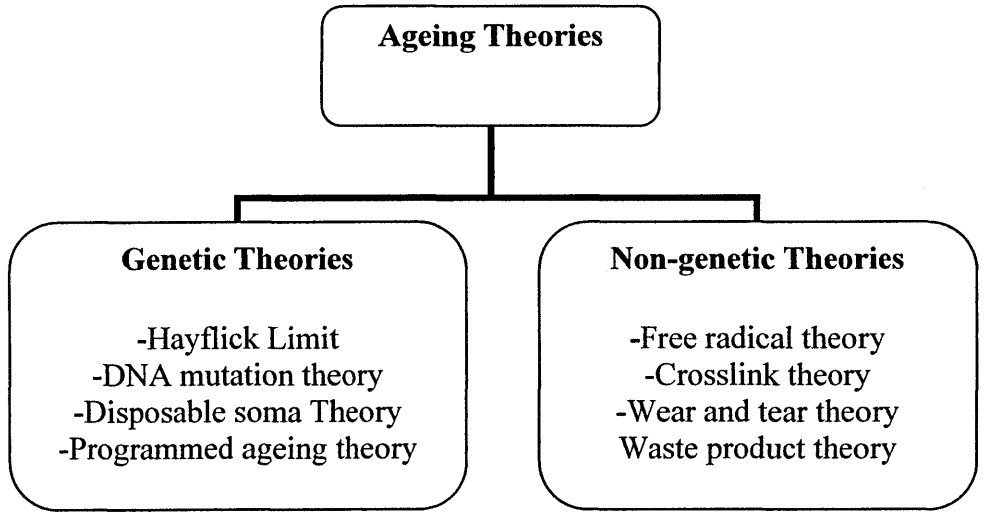


Figure 1.8: Flow diagrams showing the range of main genetic and non-genetic theories surrounding the ageing process.

Telomeres are specialised nucleoprotein complexes that have important functions primarily in the protection, replication and stabilisation of the chromosome ends. They are located at the ends of the chromosomes and prevent chromosomes becoming joined to others by effectively sealing off the end of each chromosome (Voet and Voet, 1995). Each time a cell divides the telomere length is thought to shorten and if they are not replaced then the telomere regions can reach the point where they become so short that the chromosomes become unstable, resulting in the loss of replication of essential genes located near the ends of the chromosome. The reverse transcriptase responsible for the extension and replacement of the telomere repeat sequences is telomerase (Morin, 1989) and when telomerase activity becomes diminished, which is the case in aged cells, the telomere region will gradually shorten. The shortening of telomeres has been described as a genetic clock or a fuse that is becoming shorter and shorter that eventually sets off a cellular time bomb that leads to ageing (Shay *et al.*, 2004).

The evolutionary causes of ageing include theories such as the disposable soma theory, which is based on natural selection during evolution. If we consider the fundamental unit i.e. organism to be maintained through natural selection it is not the conservation of the individual unit or the group that is important but the conservation and replication of the gene (Heylighen, 1997). Therefore, the power of natural selection allows the expression of beneficial genes for reproductive purposes but after the reproductive period and the gene has been successfully passed on to the off spring the fate of the parent organism becomes less important (Halliwell and Gutteridge, 1999).

The programmed ageing theory states that life span is directly related to the genes that we inherit and suggests that ageing begins from birth. Evidence to back this theory includes the fact that life expectancy of members from the same species are very similar; also the fact that females in several species often out live the male of the same species. Another factor is that long-lived parents tend to give rise to long-lived offspring, which again supports that longevity is determined from programmed genes passed down from the parent (Halliwell and Gutteridge, 1999). There are also several non-genetic damage accumulation theories surrounding ageing and include free radical theories, cross-linking of DNA and protein theory and the membrane hypothesis. It is largely for these reasons that oxidative stress has been explored in this present study. There are three main sources of reactive oxygen species (ROS) in cells:

(a) The major source of ROS originates by electron leakage from the electron transport chains located in the mitochondria and chloroplasts, which leads to production of ROS such as superoxide and hydrogen peroxide.

(b) The second source of ROS is from organelles called peroxisomes, which are involved in the degradation of fatty acids.

(c) The third source comes from biomolecules, and particularly secondary metabolites such as flavins and thiols, which can auto-oxidise and form ROS dependent on the antioxidant status of the cell (note that these compounds can often be both pro- and antioxidants.

In parallel to the above it is also important to consider that ROS can be produced at times when the cell's natural antioxidant and repair systems are compromised. ROS species are well documented to have deleterious effects on DNA, proteins, enzyme

activity and lipids. The free radical theory of ageing was first described by Denham Harman in 1956, when he proposed that ageing resulted from the random deleterious damage to tissues as a result of free radical damage, caused by an increase in oxidative stress (Wickens, 2001 and Kasapoglu and Ozben, 2001).

All of the non-genetic theories, the cross-link theory, the wear and tear theory and the waste product theory have one common factor and that is that oxidative stress and reactive oxygen species play a role. Cross links are formed by free radical reactions with proteins and DNA and therefore the cross link theory suggests that as a result of higher amounts of ROS there is an increased frequency of cross-linking causing long term DNA damage. The wear and tear theory refers to a decrease in the effectiveness of the antioxidant systems and a decrease in the enzymes and proteins involved in the repair of damaged biomolecules such as DNA and lipid membranes. The effect of decreased repair and antioxidant mechanisms will inevitably lead to increased ROS. The wear and tear theory is very much based on a shift in the antioxidant and prooxidant equilibrium towards the prooxidant side.

The waste product theory refers to the accumulation of by-products of oxidative reactions such as lipid peroxidation. Lipid peroxidation occurs as a result of ROS attacking and breaking down the lipid membranes, which in all cells, and are of the utmost importance in maintaining cell integrity. Lipid peroxidation results in the release of fatty acids, which then undergo further reaction giving rise to a range of secondary products. There are two very well documented products of lipid peroxidation 4-HNE and malondialdehyde, one of which is highly cytotoxic and the

other very mutagenic respectively (Adams, 1999, Di Mauro *et al.*, 1995, Fukuda *et al.*, 1997 and Esterbauer *et al.*, 1988).

Ageing in plants is, in many respects, different to the classical description of ageing in mammals in that it is often described as the deterioration of a particular organ (the leaf being the most commonly studied) rather than ageing of the entire organism (Benson, 1990). The numerous other differing characteristics between plant and mammalian systems make comparing the two directly, difficult. Plants are autotrophs and utilise carbon dioxide as their sole source of carbon; they have modular growth and development stages, which often make it difficult to determine whether a plant is alive or dead; for example, during dormancy the only non-destructive way to determine whether that seed is viable is to germinate it or to measure very low levels of metabolic activity. There is also great variation in the life-span of plants: some are seasonal and their entire life is started and finished within one year but on the other hand trees can live thousands of years and may have long dormant periods.

The function of meristems is central to the longevity of the whole plant; the complete anatomy and morphology of the plant is governed by differential cell division and cell expansion within the meristem regions. As long as the apical meristems keep producing new vegetative organs, in addition to retaining a source of "core" cells (possibly equivalent to stem cells in mammals), then the plant, and in particular the shoot structure, has an open-ended life span, i.e. the plants life span is indeterminate as long as there are functional meristems (Thomas, 2002). As a result of the vast differences between plant and mammalian organisms, senescence in plants is often discussed with respect to the leaf as it is considered a terminally differentiated, largely

autonomous entity and doing this allows a reasonable comparison between senescence in mammals and plants (Yoshida, 2003). The study of ageing in plants is very important in terms of germplasm conservation. By manipulating the environmental parameters of the storage conditions it is possible to decrease the metabolic rate and this appears to decrease the rate of tissue deterioration, which would normally be associated with natural ageing (Benson, 1990).

Although there are some fundamental differences habituation and ageing they do have several factors in common. Habituation is where the cells become independent of the PGRs and when habituated cells reach the fully habituated state they lose all totipotency and the ability to form any organised structures and proliferate autonomously as dedifferentiated cells. Aged cells maintain the need for PGRs in the medium to preserve dedifferentiation however it is common that aged cells lose their ability to undergo somatic embryogenesis. Thus although they still require PGRs to divide *in vitro* they lose the ability over time to form whole plants. Both these phenomena have one thing in common, oxidative stress, and thus by studying the antioxidant levels and extent of free radical damage in aged and habituated cells it will be possible to determine whether oxidative stress is involved and to what extent in both aged and habituated cells.

1.9 OXIDATIVE STRESS

This section will overview the possible pathways and processes that lead to oxidative stress in plants the first of starts with photosynthesis, which is primary metabolism pathway.

1.9.1 Photosynthetic processes

Photosynthesis is the process by which plants use energy from sunlight to produce sugars, which the cell then converts to ATP, the fuel used by every living organism. Photosynthesis in plants occurs in two distinct phases:

- Reactions that use light energy to generate NADPH and ATP.
- Dark reactions, that are light independent and utilise NADPH and ATP generated from the light reactions to drive the synthesis of carbohydrate from carbon dioxide and water.

The light dependent reactions occur in the thylakoid structures when light strikes chlorophyll (the principal light capturing pigment in plants) in such a manner as to excite the electrons to a higher state. Then a series of reactions, which pass energy along an electron transport chain, takes place. The electron transport chain is a series of coupled redox reactions, where electrons are passed from one protein/enzyme to the next, before being finally attached to a terminal electron acceptor which is normally oxygen or NADPH; ATP is formed as a result of the light reactions. The electron transport chain in the thylakoids resembles those found in mitochondria. In thylakoids the electron carriers involved are cytochromes, quinines and ferrodoxin. The dark reactions are the light independent reactions which use NADPH and ATP to drive the synthesis of carbohydrate (via the Calvin cycle) as a source of energy for the plant (Lawrence, 2000). There are three main steps in the Calvin cycle, which include carbon fixation utilising ribulose-1-5-biphosphate (RuBP), 3-phosphopglycerate (PGA) conversion to 3-phosphoglyceraldehyde (PGAL) and finally conversion of PGAL into carbohydrate (see Figure 1.9).

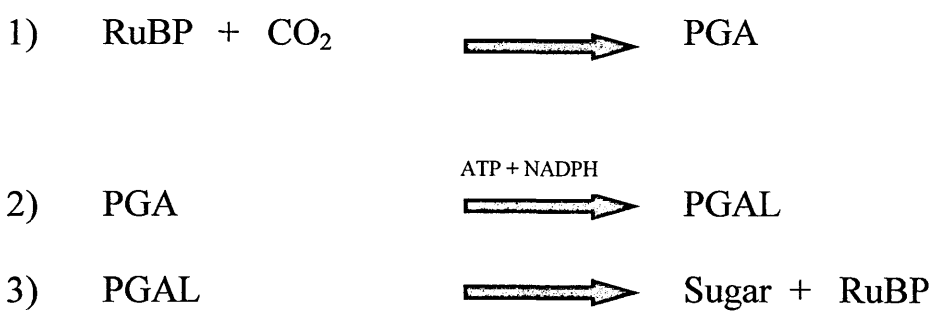


Figure 1.9: Diagram showing the three chemical conversions that take place in the Calvin cycle during photosynthetic dark reactions. Step one: Carbon fixation reaction converts RuBP to 3-phosphoglycerate. Step two: Conversion of 3-phosphoglycerate, at the expense of ATP and NADPH, yields 3-phosphoglyceraldehyde. Step three: 3-Phosphoglyceraldehyde is converted into one molecule of sugar and five molecules of RuBP, which is looped back into the start of the cycle.

During carbon fixation carbon dioxide is combined with RuBP to create PGA molecules. PGA conversion uses energy (ATP and NADPH) created during the light reactions to convert PGA to PGAL. Finally one of the six PGAL molecules that are produced is converted into a sugar molecule and the remaining five are converted back to RuBP, which is then fed back into the Calvin cycle (Tiaz and Eduardo, 2002).

1.9.2 Plant respiration

The majority of energy in plants is obtained autographically through photosynthesis; however plants need a supply of energy "in the dark" and so they also have mitochondria where energy can also be produced through respiration. In the case of plant tissue cultures the cells are maintained heterotrophically in the presence of organic carbon substrate which is most commonly sucrose and/or in some systems glucose.

Plant mitochondria carry out the Krebs cycle in the matrix and electron transport chain reactions in the unfolded cristae of the inner membrane (Tiaz and Eduardo,

2002). The electron transport chains pump protons into the inter membrane space and this results in a gradient being formed. When the protons pass back through to the matrix they do so through a ATP synthase membrane complex and the energy is trapped resulting in ATP formation. Plant mitochondria have a similar structure to mammalian mitochondria in that they contain the classical complexes I (NADH dehydrogenase), II (succinate dehydrogenase), III (cytochrome c reductase) and IV (cytochrome c oxidase), however in addition, it has been found that plants contain at least five other complexes, which are involved in respiration. These alternative oxireductases do not pump protons across the membrane and their role in plants has sparked considerable interest (Eubel *et al.*, 2004). In *in vitro* tissue culture, as there is a source of organic carbon supplemented in the media, plant cells do not need to carry out photosynthesis. However light is often still a requirement for morphological development.

Electron transport chain reactions are under very tight metabolic control; molecular oxygen sequentially accepts electrons leading to its final reduction where ATP is produced to drive metabolic processes. Oxygen is normally reduced to water by two enzymes in the electron transport chain but when electrons escape from the transport chain, then oxygen will only be reduced by one electron, giving rise to superoxide radicals, which can if not detoxified lead onto the formation of a range of other ROS (Davies, 1995). A form of metabolic disruption normally causes electron leakage. ROS can also be generated in a variety of other ways; the environment produces ROS from natural sources such visible light, ultraviolet light and ozone, and in addition ROS, can be produced from manmade sources such as car and industrial emissions (Madhumanjari and Mukherji, 2001).

In *in vitro* tissue culture, the environment is controlled and the air is very clean, thus the majority of ROS are formed as a by-product of a disruption in normal aerobic respiration, often caused in response to cell stress. Under typical metabolic conditions around 5% of molecular oxygen consumed by mitochondria in mammalian cells is converted into ROS (Halliwell and Gutteridge, 1999).

1.9.3 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) can be divided into two main types: free radical ROS and non-radical ROS, with the former being the more reactive. To appreciate the reactivity of free radical species it is important to understand their chemical nature. Bond formation between molecules is dependent on the interaction of paired electrons in the outer shells. Free radicals, defined as species that contain one or more unpaired electrons in its outer electron shell, however do not follow the normal rules of bond formation (Benson, 1990; Halliwell and Gutteridge, 1999). The unpaired electron makes free radical species extremely reactive as they have a high chemical drive to pair the electron. They quench their activity by abstracting an electron from any molecule in the vicinity that is capable of forming a bond and it is often the case that the radical species reacts with a biomolecule component.

ROS formation is caused by various physical and chemical factors in living organisms. There are several different sources of ROS in living organisms including endogenous routes such as ROS production from the respiratory chain in mitochondria (Boveris, 1984, Halliwell and Gutteridge, 1999), from the leakage of electrons from electron transport chains in the photosystems in chloroplasts (Asada

and Takahashi, 1987), and from peroxisomes (Gille and Sigler, 1995). Exogenous sources include reactions of ozone (Farage *et al.,* 1991) and ionising and UV radiation (Halliwell and Gutteridge, 1999). It is important to realise that not all active oxygen species are free radicals, however are still produced in an oxidative reaction or formed as a secondary product of a previous free radical reaction and are also capable of reacting with organic biomolecules (see Table 1.5 for overview of main ROS formed).

Reactive oxygen species are capable of rapid and non-specific reactions and can cause lipid peroxidation, inactivation of enzymes and other functional proteins, DNA modifications and DNA strand breaks (Bartosz, 1997). The involvement of ROS in several different human diseases including Alzheimer's disease (Lyras *et al.,* 1997), Parkinson's disease (Cohen, 1984), AIDS (Flores *et al.*, 1993) and cancer (Senthil *et al.*, 2004, Sanders *et al.*, 2004, Lopaczynski and Zeisel 2001 and Halliwell, 1996) has been well established,.

Free Radical Reactive Oxygen Species		**Non-radical Reactive Oxygen Species**	
Superoxide	$O_2^{\bullet-}$	Hydrogen peroxide	H_2O_2
Hydroxyl radicals	$OH^{\bullet}$	Ozone	O_3
Peroxyl	$RO_2^{\bullet}$	Singlet oxygen	$^1\Delta gO_2$
Alkoxyl	$RO^{\bullet}$	Reactive chlorinating species	^-OCl
Hydroperoxyl	$HO_2^{\bullet}$	Reactive nitrogen species	$ONOO^-$

Table 1.5: Main reactive oxygen species found in living organisms. The partial reduction of molecular oxygen results in the formation of superoxide radicals, hydrogen peroxide and the highly reactive hydroxyl radical. Catalysed reaction of hydrogen peroxide with chloride anions gives rise to reactive chlorinating species and the reaction of superoxide radicals with nitric oxide yields reactive nitrogen species.

Oxidative stress is initiated by a shift in the prooxidant and antioxidant equilibrium. The extent of ROS production and subsequent damage to biomolecules is very much dependent on the effectiveness of the antioxidant systems in place to detoxify the ROS, and also the systems that are available to repair oxidative damage (Bartosz, 1997). There are several enzymes, including superoxide dismutase (SOD), catalase, peroxidase and glutathione S-transferase involved in the direct removal of ROS and several other molecules, such as glutathione and ascorbate, which play a role in ROS removal (Halliwell and Gutteridge, 1999) (antioxidant systems will be discussed in further detail in section 1.11).

The formation of ROS often begins from reactions involving molecular oxygen and takes place in electron transport chains found in mitochondria and chloroplasts. Although molecular oxygen is relatively unreactive it is utilised by biological systems in respiration processes. Cytochrome oxidase adds four electrons to molecular oxygen in a series of tightly regulated redox reactions to form water and the energy (ATP). However, during metabolic disruption superoxide radicals ($O_2^{\bullet -}$) can be formed when only one electron is added to molecular oxygen (Bartosz, 1997).

$$e^- + O_2 \longrightarrow O_2^{\bullet -}$$

Superoxide is a charged species, which means that this molecule cannot diffuse through membranes. In addition to the damage that this radical can itself cause, it can also undergo further reactions within the cell giving rise to hydroxyl radicals and singlet oxygen; therefore *in situ* detoxification is essential (Alscher *et al.*, 1997). Superoxide dismutase (SOD) is the enzyme involved in the removal of superoxide

radicals; there are several different types of SOD's grouped depending on their associated metal ion. The various different forms of SOD will be discussed in more detail later in this chapter. SOD is found in high concentrations in the mitochondria and chloroplasts, the sites of electron transport chains (Halliwell and Gutteridge, 1999). Superoxide radicals are converted by SOD dismutase into hydrogen peroxide (H_2O_2), which although not a radical species, is also usually considered an ROS.

Hydrogen peroxide is a weak oxidising compound and is thought to play a crucial role in plant cell signalling. Burdon *et al.* (1995) demonstrated that hydrogen peroxide was capable of promoting growth responses in mammalian cells (Burdon, 1995) and more recently similar responses were found in plants (Kairong, 1999). Somatic embryogenesis (production of embryo structures from somatic cells) is a very important process during the early development of plants. The proteins that are involved in somatic embryogenesis vary in timing, the period, and extent of expression (Kairong *et al.,* 2002). Exogenous hydrogen peroxide treatment improved the frequency of somatic embryogenesis in *Lycium barbaium* L, by increasing the synthesis of proteins by increasing protein phosphorylation (Van Breusegem *et al.,* 2001, Suzuki *et al.,* 1997). Since hydrogen peroxide can be generated by a large variety of cell types and has the ability to penetrate cell membranes very rapidly this supports the suggestion that hydrogen peroxide plays a role in plant development.

The concentration of hydrogen peroxide in normal embryogenic cells is generally below 100mM (Kairong *et al.,* 2002). It was also demonstrated during the same study that when hydrogen peroxide concentration exceeded 100mM somatic embryogenesis was inhibited suggesting that hydrogen peroxide acts as a cellular messenger below a

critical concentration. Above that concentration, normal cellular growth responses are inhibited and hydrogen peroxide can rise to levels that can prove lethal to the cell by promoting and taking part in ROS reactions. Hydrogen peroxide can also take part in a series of reactions, which lead to the formation of the highly reactive hydroxyl radical ($HO^{\bullet}$). The reaction is known as the Haber-Weiss reaction and involves the interaction of hydrogen peroxide and superoxide radicals in the presence of a proton donor, to give oxygen and the hydroxyl radical ($HO^{\bullet}$).

Haber-Weiss reaction

$$H_2O_2 + O_2^{\bullet-} \longrightarrow O_2 + HO^- + HO^{\bullet}$$

Overall the above reaction is really a summation of two other reactions involving iron ions. In Equation 1, iron, in the form of ferric ions, catalyses the conversion of superoxide into oxygen and the ferrous ion. Equation 2 shows the reaction of this ferrous ion with hydrogen peroxide to form hydroxyl radicals and regenerated ferric ions (the Fenton Reaction) (Benson, 1990).

1) $Fe^{3+} + O_2^{\bullet-} \longrightarrow Fe^{2+} + O_2$

2) $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + HO^{\bullet}$

The hydroxyl radical is the most reactive of the free radical species and is capable of damaging a wide range of cell components therefore its immediate *in situ* removal is absolutely essential (Alscher *et al.,* 1997). Antioxidants such as glutathione and

ascorbate can quench hydroxyl activity, but it is so reactive that very often the damage is done before the radical is detoxified. The rate of reaction of the hydroxyl radical is diffusion controlled and it reacts very close to its site of production, therefore damage caused by this radical is very site-specific (Hermández *et al.*, 1995).

Another ROS that is considered to cause considerable damage to biomolecules is singlet oxygen ($^1O_2$), which is a form of partially oxidised oxygen. When energy is applied to molecular oxygen it undergoes spin reversal, which results in an oxygen with paired electrons in the reactive orbital. Although singlet oxygen is not a free radical, its unusual electronic arrangement gives it several free radical properties. It is capable of damaging several biomolecules, in particular lipids; therefore lipid membranes are primary targets for singlet oxygen. Nucleic acids are also susceptible to singlet oxygen attack (Cadet *et al.*, 1995). There are several other ROS that can be formed, many of which are products of the primary ROS attack such as lipid hydroperoxides and carbohydrate reactive species, which will be discussed in more detail later on in this chapter (see section 1.10). Overall, the generation of these ROS are often only the beginning in a series of events, which may result in a cascade of free radical and oxidative reactions, giving rise to secondary oxidative products.

Highly reactive oxidants, such as the hydroxyl radical are capable of reacting with DNA, RNA, lipids, proteins, carbohydrates and several antioxidant species (see Figure 1.10). Other oxidants such as hydrogen peroxide and lipid peroxides can be much less reactive and also much more target specific. There are five main factors that determine the rate of reaction between biomolecules and oxidants: the

concentration of the target site, the rate constant of the oxidant to the target, the location of the target in relation to the oxidant production, the extent and occurrence of secondary events that take place as a result of free radical chain reactions and, finally, the efficiency of the antioxidants in the vicinity (Davies, 2005).

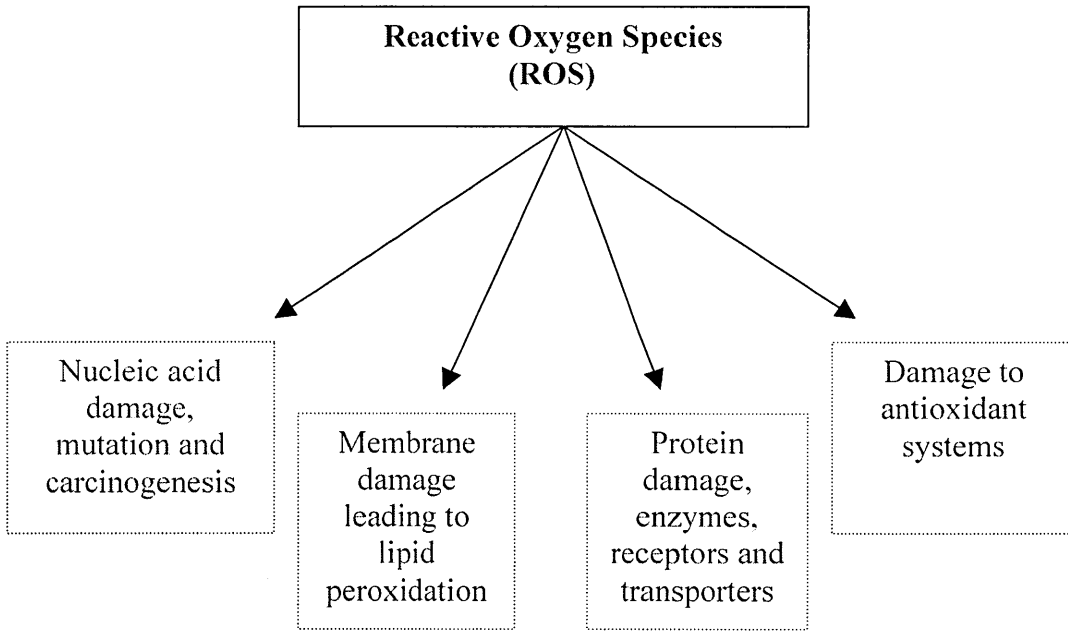


Figure 1.10: Diagram showing an overview of the range of biomolecules that can be damaged by reactive oxygen species in a prooxidant environment.

1.9.4 Hypersensitive response: ROS mechanisms against pathogen attack

Whilst ROS can be considered to be highly destructive, it is also important to consider that they can be used by plants in their pathogenic defence systems. Whereby the plants employ the destructive processes of ROS to attack invading pathogens. This is a similar process to the use of ROS by animal blood cells as a defence against pathological diseases. The hypersensitive response is a universal phenomenon found in plants that is designed to protect cells from attack by incompatible pathogens. These strategies are designed to restrict the invading pathogen to the site of initial

penetration. Resistance is achieved through a combined effect of two processes, hypersensitive resistance (HR) and local acquired resistance (LAR) (Ghannam *et al.*, 2005, Dorey *et al.*, 1997). The HR response has been shown to occur in plants in reaction to viral, fungal and bacterial pathogens (Dangl and Jones, 2001). HR is activated when the plant resistance gene recognises a specific pathogen's avirulence gene (avr gene), which then results in rapid and transient responses that occur at the plant's surface with the end outcome being rapid, localised host cell death (Dorey *et al.,* 1997). During HR it has been reported that there are changes in ion-flux, protein phosphorylation and de-phosphorylation, changes in the exocellular PH, variation in membrane potentials and rapid production of ROS, or "oxidative burst" as it is commonly termed (Wojtaszek, 1997a).

The localised acquired resistance (LAR) occurs in the living cells that surround the HR lesion. LAR activates defence responses in the surrounding cells, which make it a highly inhospitable environment for the invading pathogen (Ghannam *et al.,* 2005). LAR does not cause cell death, however it is very tightly connected with HR. HR is thought to be stimulated by the exogenous signal from the invading pathogen (Dorey *et al.,* 1997); whereas LAR is induced by an endogenous signal produced during the onset of HR (Ghannam *et al.,* 2005). HR coincides with a number of other metabolic changes, such as strengthening of the structural barriers, synthesis of lytic enzymes and accumulation of pathogenesis related (PR) proteins. These processes are all part of the increased systemic resistance to prevent the pathogen attacking (Zhang *et al.,* 2004).

There are two main hypotheses surrounding the origin of ROS, during the onset of HR. Firstly, it was suggested that ROS were generated from reactions involving NADPH oxidase (Wojtaszek, 1997b). Secondly, they are generated from the pH-dependent formation of ROS via cell wall cationic peroxidase (Bolwell and Wojtaszek, 1997). However the pathways are thought to be plant and, possibly, pathogen dependent (Hammong-Kosack and Jones, 1996) and there are also probably several other sources of ROS, which have not been fully elucidated. The first ROS formed as a result of initiation of oxidative burst is the superoxide radical, which having a very short half life and cannot easily pass through membranes, are normally converted into other oxidative species (Alscher *et al.,* 1997). Superoxide radicals can undergo dismutation to form hydrogen peroxide (H_2O_2) or they can be protonated to form hydroperoxyl radicals ($HO_2\cdot$).

Hydrogen peroxide itself is not highly reactive but the theory is that, as it can pass through membranes very easily, it diffuses through to the nucleus of either the plant or pathogen and undergoes Fenton reactions in the presence of Fe^{2+} ions to form the highly destructive hydroxyl radicals (Hammong-Kosack and Jones, 1996). Hydroperoxyl radicals are uncharged species, which like hydrogen peroxide can diffuse through the membranes with relative ease and can directly attack fatty acids leading to the production of lipid hydroperoxides (see Figure 1.18), which can go onto form signal molecules such as jasmonates (implicated in programmed cell death in plants) or highly cytotoxic secondary products such as 4-HNE.

Recent research surrounding the hypersensitive response has lead to the identification of signal transduction pathways, which appear to couple the pathogen recognition

pathway to the defence response. The pathways include three different signal molecules: salicylic acid (SA), jasmonic acid (JA) and ethylene (Glazebrook, 2001, Hammong-Kosack and Jones, 1996, Zhang *et al.,* 2004). These signalling pathways can be divided into two groups, an SA dependent pathway and an SA independent pathway, which involves JA and ethylene (Kunkel and Brooks, 2002). These two pathways, although different, do not function completely independently of each other and there is evidence to suggest that they influence each others regulatory interactions (Glazebrook, 2001). Salicylic acid has been implicated in plant defence pathways and it has been shown that SA levels increase in plant tissue following a pathogen infection and the exogenous application of SA in tissue culture increased resistance of plants to several pathogens (Ryals *et al.*, 1996). It was also shown that increased SA production was necessary for the expression of resistance genes, which aid the establishment of the systemic acquired resistance.

Jasmonic acid is a fatty acid-derived signalling pathway, which like SA is involved in inducing defence mechanisms. It is thought that there is much interaction between JA and SA and there is evidence for both negative and positive correlations between these two processes (Kunkel and Brooks, 2002). The inhibitory effect that SA has on tomato is very well documented (Doares *et al.,* 1995) and also there was an inverse correlation between JA and SA during the initial wound response in rice (Lee *et al.,* 2004). The effects of JA and SA on each other appear to be very much dependent on the plant species (Kunkel and Brooks, 2002). Jasmonic acid has been shown to be directly involved in a number of defence mechanisms, including the rapid appearance of defensive proteins (jasmonate inducible proteins, JIP's) (Glazebrook, 2001) and a

number of metabolic changes including acceleration of senescence processes (Ueda and Kato, 1980).

Ethylene's role in plant defence has not been fully elucidated; ethylene has been shown to have both an inhibitory effect in Arabidopsis infected by *Botrytis cinerea* (Thomma *et al.,* 1999), however the opposite was the case in Arabidopsis infected with *Pseudomonas* and *Xanthamonas* pathogens (Bent *et al.*, 1992). The evidence suggests that ethylene's role in defence processes may be pathogen dependent.

1.10 REACTION OF OXIDANTS WITH BIOLOGICAL TARGETS

1.10.1 Proteins

Proteins are essential in living organisms, since enzymes and structural components comprise proteins and they play a crucial role in the control of gene expression. All proteins are made from a linear chain of a combination of 20 known amino acids, which are joined by covalent peptide bonds. The number and sequence of the amino acids used, determine the structure and function of the completed protein. The manner in which the protein is folded forms an active site at which the desired chemical reaction takes place. Exactly how the linear chain of amino acids folds into its final three-dimensional structure is not fully understood. However, it is clear that proteins can fold into secondary, tertiary and quaternary levels of structure (Voet and Voet, 1995).

Proteins constitute 75% of the combined mass of potential initial molecular targets for ROS in eukaryotic species (Alberts *et al.*, 2002) while the other 25% comprises of lipids, DNA and other biomolecules. Due to their abundance in living systems it makes them prime targets for oxidation. Until recently, protein oxidation was not a well-researched area due to its complexity because of all the different side group conformations and the potential products. In addition, there has been a lack of sensitive, stable markers of protein damage so previous studies mainly focussed on lipid and DNA oxidation (Davies, 2005). Protein oxidation can occur in two places: the backbone or on the amino acid side chains. The location of the oxidative damage depends very much on the nature of the ROS and the concentration of the antioxidants in the area (Grune *et al.*, 2003).

Highly reactive oxidants, such as hydroxyl radicals, are capable of reacting with the backbone of the protein structure and these reactions cause the most damage often leading to protein fragmentation through a series of reactions as follows: radicals react rapidly with the α-carbon site (carbon with the amino acid side chain attached) causing the production of a carbon centred radical species. This radical can react with another radical or, most commonly, with oxygen giving rise to peroxyl radicals. Peroxyl radicals then eliminate $HO_2\cdot$ to generate an imine and then, through hydrolysis, lose water resulting in fragmentation of the protein backbone.

Non-radical oxidants cause very little damage to the backbone of the protein but can react with amino acid residues on the side groups. Singlet oxygen, for example, has been shown to selectively target and oxidise tryptophan, tyrosine, histidine, methionine and cysteine residues (Wilkinson *et al.*, 1995, Davies, 2003). The above

amino acids are selected by electron deficient oxidants because they are electron rich. The effects of protein oxidation can be very serious since, as mentioned earlier, the function of the protein is very dependent on the correct structure. Fragmentation caused by reactive radical species results in protein damage leading to loss of some or all protein function (Davies, 2005). It was demonstrated that when the residue methionine was oxidised by calmodulin, there was significant conformational changes that resulted in the loss of protein stability (Gao *et al.,* 1998).

Chain reactions taking place after initial protein oxidation are also another problem; oxidised proteins are often reactive and can undergo further reactions with the same protein or other biomolecules in the vicinity (Dean *et al.,* 1993). Protein oxidation contributes to the pool of damaged enzymes and the size of this pool increases with age and various pathological conditions. Oxidised protein accumulation may simply be a case of protein production rates being higher than the rates of degradation; however the causes are not entirely clear. It was suggested that DNA damage caused during periods of oxidative stress may adversely effect the concentration or activity of some of the factors involved in the rate of production or the rate of degradation (Stadtman, 1992).

A proteolytic complex was discovered in 1980 by Wilk and Orlowski, which can selectively identify and degrade damaged proteins. It is proposed that the proteasome recognises any exposed hydrophobic amino residues, which are normally tucked inside the tertiary or quaternary structures (Grune *et al.,* 1997). However, if the protein is more damaged (to the extent of two or more amino acid modifications) then the proteasome does not recognise the site, probably due to extensive cross-linking

and aggregation which renders them less susceptible to proteolytic degradation (Davies, 2003). The oxidation of methionine residues has been suggested to trigger the protein turnover; however the reaction appears to be dependent on the extent of oxidative damage (Grune *et al.,* 1997). Ferrington *et al.* (2001) demonstrated that calmodulin when oxidised at the methionine residue increased protein turnover and degradation, however when the hydrophobicity of the protein was altered due to more extreme oxidative damage the protein turnover was not increased (Ferrington *et al.,* 2001). Cells attempt to repair the modifications to restore the original functions. Some of the proteins involved in this process are also known as "chaperone" proteins and their function is to aid in the refolding of the protein structures (Bose *et al.,* 1996). Like the degradation mechanism, this group of proteins are only capable of repairing moderately damaged proteins, when only protein refolding is required (Grune *et al.,* 1997).

1.10.1.1 Sulphydryl groups

Sulphydryl groups can be divided into two groups, low molecular weight species such as glutathione (GSH) and high molecular weight sulphydryls (protein-bound sulphydryl groups). Protein-bound sulphydryls are located on protein side chains and can form an active site in an enzyme or, in membranes, can provide a barrier against ROS attack. Oxidation of sulphydryl groups as a result of ozone exposure leads to changes in membrane structure (Chevrier, 1988). Changes in the permeability and ionic balance results in disturbance of many of the metabolic processes, which ultimately leads to a decrease in cell division, cell growth and development (Chevrier, 1988). Sulphydryl groups have also been shown to protect the mitochondrial

complexes from damage from the highly cytotoxic, lipid peroxidation product 4-hydroxy-2-nonenal (Korotchkina *et al.,* 2001).

1.10.2 Lipid peroxidation

Lipids are found in all living organisms and include fats, oils, fatty acids, glycolipids, phospholipids and steroids. Phospholipids are a group of amphipathic lipids with a glycerol or sphingosine backbone. They have fatty acid side chains and a phosphorylated alcohol head group (see Figure 1.11). When phospholipids are placed in water they tend to aggregate to form micelles, when the polar heads are placed in water they interact with water and the non-polar alkyl tails intermingle with each other. There are two types of micelle formation: spherical and bilayer. The bilayer is the more important of the two structures as it forms all membranes within the cell. Lipid bilayers are comprised of a double layer of phospholipids orientated so that their hydrophilic head is towards the outside of the layers and their hydrophobic tails are tucked away in the inside (see Figure 1.12).

When the lipid bilayer is attacked by ROS, cell associated enzymes known as lipases are activated and these enzymes attack the lipids at the C-2 position of the glycerol backbones, causing the breakdown of the lipid and the liberation of the polyunsaturated fatty acids (PUFA's), which were originally the side groups of the lipids (Spiteller, 2001). Polyunsaturated fatty acids are prone to further attack from ROS and can undergo a number of cascading reactions, producing intermediate ROS that can abstract hydrogen from the reactive methylene group on the PUFA, leading to the formation of a dienyl radical (Moore and Roberts, 1998). The dienyl radical is a

short-lived species and, after bond rearrangement, forms a conjugated diene. Conjugated dienes can uptake oxygen to form a peroxyl radical, which is unstable, short-lived and this species abstracts another hydrogen from the PUFA forming lipid hydroperoxides (Moore and Roberts, 1998).

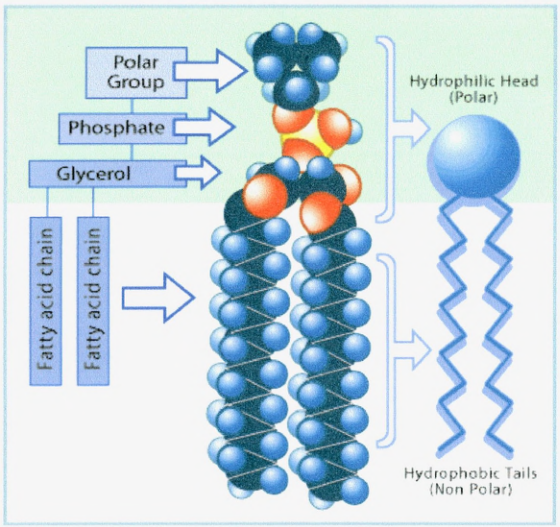


Figure 1.11: Diagram showing the structure of phospholipids which belong to the amphipatic lipid group having a glycerol backbone and a phosphorylated alcohol hydrophilic head group. Attached to the backbone on the opposite side from the head group are the fatty acid hydrophobic tail groups. Image obtained from http://bioteach.ubc.ca, artist Jane Wang, permission obtained.

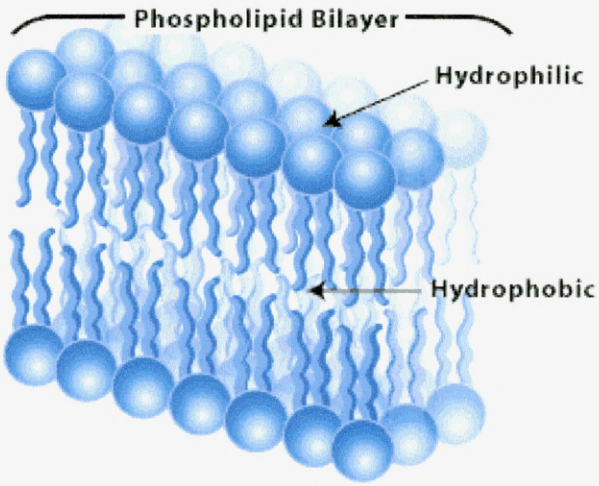


Figure 1.12: Diagram showing the structure of a lipid bilayer. The double layer of molecules is formed by phospholipids in an aqueous environment. Each phospholipid is orientated with its hydrophilic head group on the outside and the hydrophobic tails towards the inside of the structure. This diagram was obtained from www.jadross.mcmail.com. Image obtained from http://bioteach.ubc.ca, artist Jane Wang, permission obtained.

1.10.2.1 Lipid hydroperoxides

Lipid hydroperoxides can be converted, via an enzymatic pathway (see Figure 1.17) into their acid derivatives, which, in mammals, is most often arachidonic acid widely recognised as a major signalling molecule (Spiteller, 2001). Plants do not produce arachidonic acid but rather, linolenic acid, which is then converted to jasmonic acid (Spiteller, 2001). Jasmonates are widely distributed in plants and like other plant growth regulators are active at relatively low concentrations (Engvild, 1989). They have been shown to be involved in the signalling pathways that regulate the expression of the plant defence genes (Koda, 1997). Jasmonic acid can induce a physiological change or can induce programmed cell death (see Figure 1.17) (Spiteller, 2002).

Non-enzymatic lipid peroxidation leads to an increase in ROS and toxic secondary products of lipid peroxidation as a result of cell lysis, which causes damage to neighbouring cells and an overall increase in ROS (see Figure 1.17). This switch between enzymatic and non-enzymatic lipid peroxidation is associated with the change from controlled cell death, (apoptosis) to necrosis (Spiteller, 2002). The switch between the two forms is associated with oxidative damage, whereby the level of ROS overwhelms the protective systems and necrosis begins (Esterbauer, 1996). Increased ROS also causes alterations in the redox potential, which produces a change in the membrane permeability, leading to an influx of calcium ions causing permanent membrane alterations (Jabs, 1999). Ageing, cell injury and cell proliferation all cause changes in the cellular membrane (Spiteller, 2002).

1.10.2.2 Secondary products of lipid peroxidation

When lipid hydroperoxides undergo β-cleavage reactions secondary ROS are formed. Aldehydes, such as acrolein, malondialdehyde, 4-hydroxy-2-nonenal and dienals are among the products; some are highly toxic and many are potent electrophiles capable of reacting with a variety of biomolecules. α, β-Unsaturated aldehydes are among the secondary products which have a carbon-carbon double bond conjugated with an aldehydic carbonyl carbon group (Witz, 1989). The simplest of the α,β-unsaturated aldehydes is acrolein (see Figure 1.13) though 4-hydroxy-2-nonenal (4-HNE) is the most documented of the α,β-unsaturated aldehydes (see Figure 1.14). Lipid peroxidation and 4-HNE production is implicated in a number of pathological conditions including cancer and atherosclerosis (Witz, 1989 and Esterbauer, 1988).

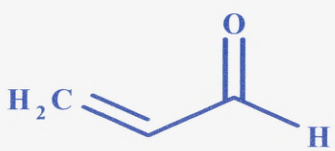


Figure 1.13: Acrolein

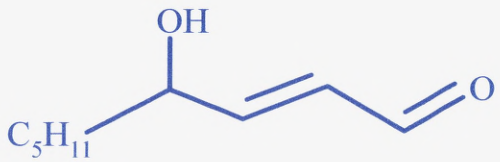


Figure 1.14: 4-Hydroxy-2-nonenal

4-Hydroxy-2-nonenal is considered to be the most cytotoxic aldehyde produced during lipid peroxidation and can cause a range of biochemical and genetic changes that are detrimental to cells (see Figure 1.17) (Esterbauer *et al.,* 1988 and Witz, 1989). In plants, 4-HNE is derived from the polyunsaturated fatty acid known as linoleic acid (see Figure 1.15). Malondialdehyde (MDA) is another common aldehyde produced as a result of lipid peroxidation and is derived from linolenic acid (see Figure 1.16). MDA is the most abundant aldehyde produced and it is also the most mutagenic formed. MDA has two functional carbonyl groups, which are capable of forming

multiple, potentially lethal DNA lesions (Marnett, 1999). Due to the structural differences between MDA and 4-HNE they accumulate in different areas of the cell. MDA has the shorter chain length; thus it can pass through membranes easier, which accounts for the higher mutagenicity of MDA. Hydroxyalkenals and specifically 4-HNE is a nine carbon unsaturated aldehyde, it has both a lipophilic and hydrophilic end and it tends to accumulate around the cell membrane area. Many important enzymes are attached or embedded in the cell membrane and 4-HNE can cause considerable damage, resulting in inactivation of many membrane-associated enzymes and further peroxidation (Witz, 1989).

DNA polymerases (α and β) are essential enzymes involved in the regulation of cell division and 4-HNE has been shown to bind to these enzyme-functional sulphydryl groups and inhibit enzyme activity (Poot *et al.*, 1988). This results in a decrease in DNA replication and therefore cell proliferation. The addition of 4-HNE to culture medium has been shown to have direct detrimental effects of proliferation of Daucus *carota* (Adams, 1999). 4-HNE and MDA react readily with thiol containing compounds such as glutathione (Poot *et al.,* 1987). When 4-HNE was added to a cell culture with foetal calf serum, which contains proteins with functional SH groups, over 90% of the 4-HNE was bound to the protein SH groups within 1hr (Kaneko *et al.,* 1987).

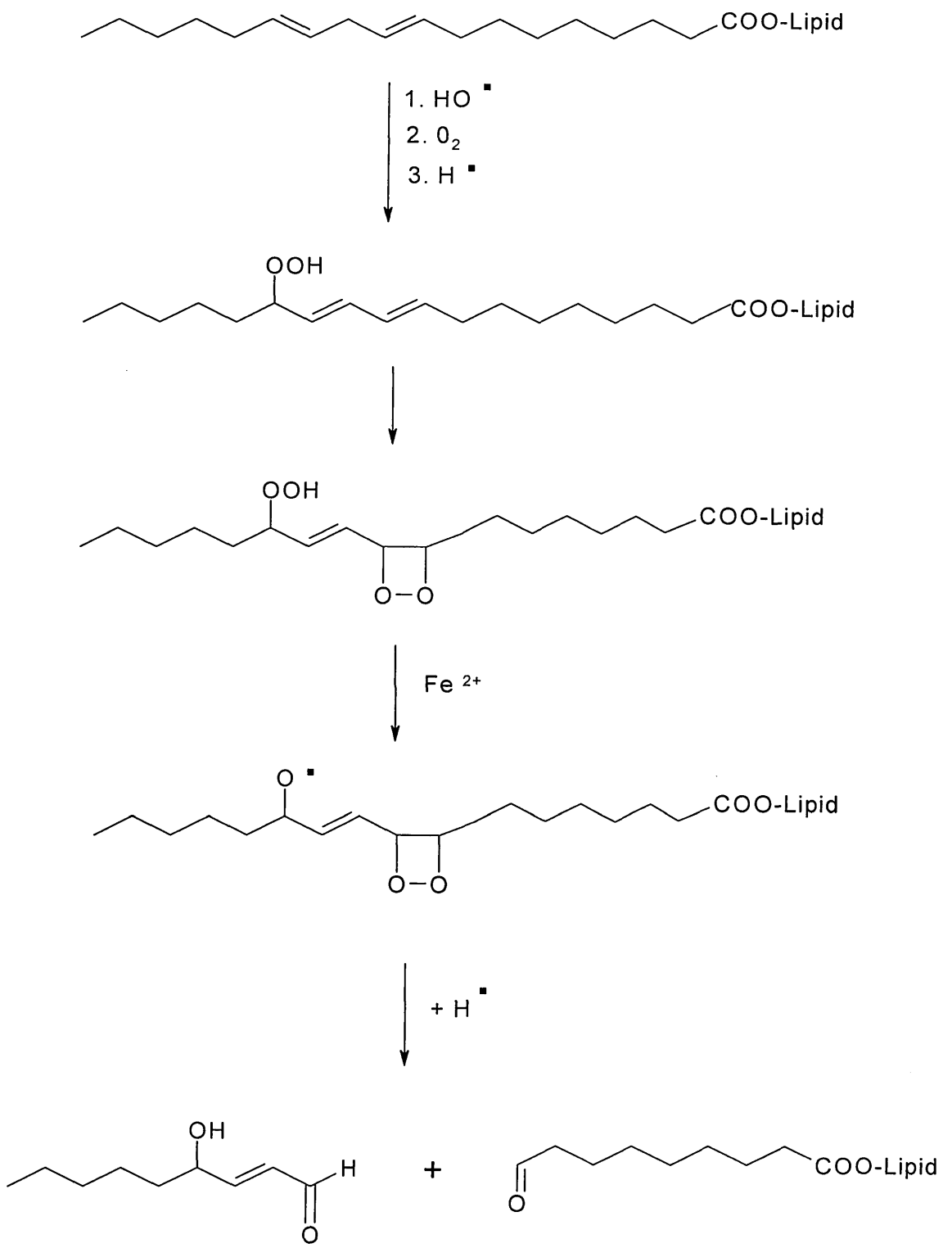


Figure 1.15: Formation of 4-hydroxy-2-nonenal (4-HNE) from linoleic acid

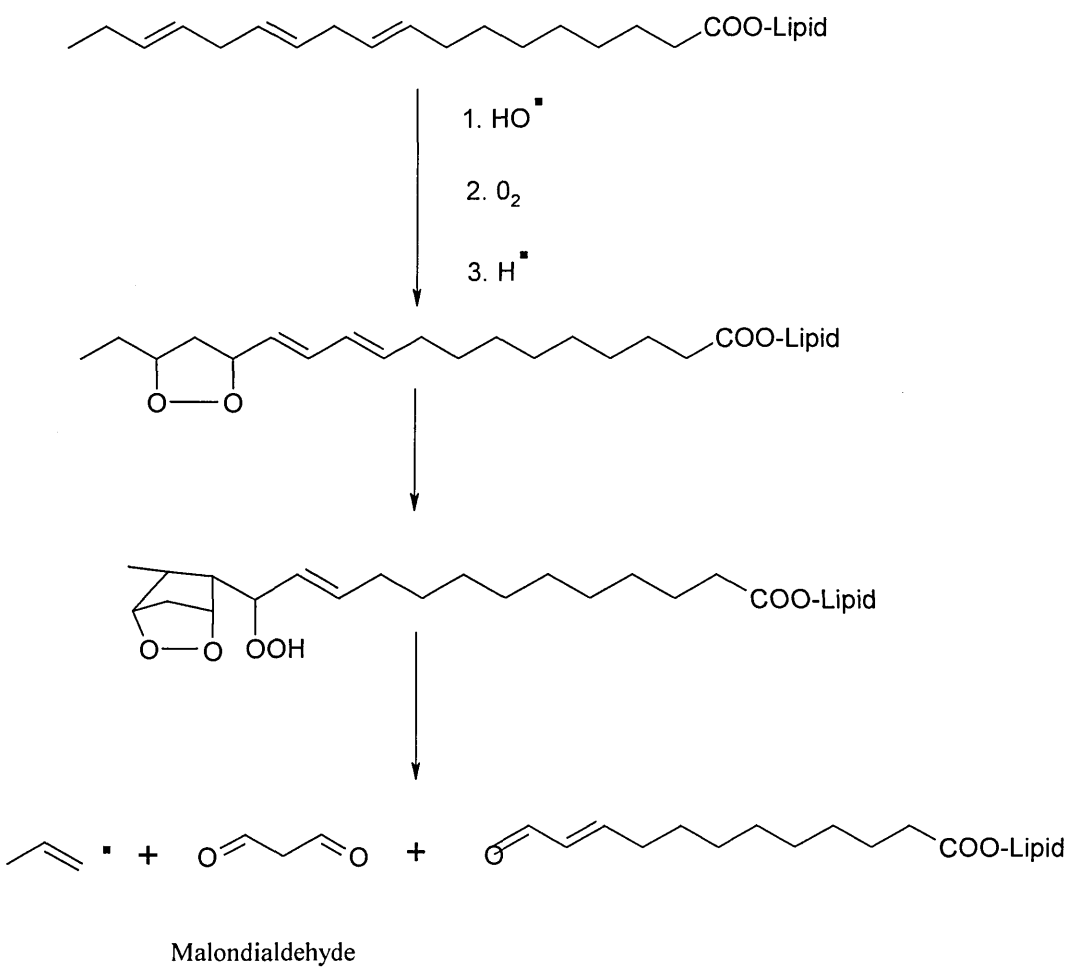


Figure 1.16: Formation of malondialdehyde (MDA) from linolenic acid

The addition of 4-HNE to mammalian cultures has been shown to increase glutathione S-transferase (an enzyme responsible for 4-HNE removal) activity by over 600 times and this also leads to a rapid decrease in intracellular glutathione levels (Siems *et al.*, 1997). Both 4-HNE and MDA can be detoxified by glutathione S-transferase, which conjugates the aldehydes to glutathione and the resulting products are then removed from the cells by the glutathione pumps located in cell membranes (Zollner, 1991). 4-HNE and MDA can also cross-link with proteins and DNA to form Schiff's bases. The accumulation of MDA and 4-HNE can lead to long-term damage and, in particular, genetic instability.

Plant cells appear to be far more sensitive to the effects of 4-HNE and MDA compared to that of animal and microbial species. Thus, when 4-HNE and MDA were added exogenously to plant cell cultures detrimental effects on cell proliferation were observed at nM/L concentrations (Adams, 1999), whereas animal (Poot *et al.*, 1988) and microbial cultures (Turton *et al.*, 1997) were tolerant to μM/L or in some cases mM/L concentrations. Interestingly, in plant cells 4-HNE and the effect on cell proliferation was not dose dependent and, in fact, cell proliferation was inhibited more at a lower dose (Adams, 1999).

Free radical activity is elevated during morphological development of cells (Benson *et al.*, 1992). The levels of 4-HNE were shown to increase in cultures that were embryogenic compared to those that were non-embryogenic. MDA was found to have a lower concentration in embryogenic cultures and a higher concentration in the non-embryogenic cultures (Bremner *et al.*, 1997). It has already been established that the effects of 4-HNE and MDA on plant cells are seen at relatively low concentrations and, consequently, it is essential that these compounds are not allowed to accumulate in the tissues. This is where glutathione S-transferase is thought to play a very important role. Glutathione S-transferase has been shown to be inhibited by the accumulation of glutathione conjugates (Zollner, 1991), so the emphasis is on the glutathione pumps embedded in the cell membranes to remove the glutathione conjugates that are formed between 4-HNE, MDA and glutathione. The efficiency of the glutathione pumps is affected by a decrease in the redox potential, which can be caused by oxidative stress.

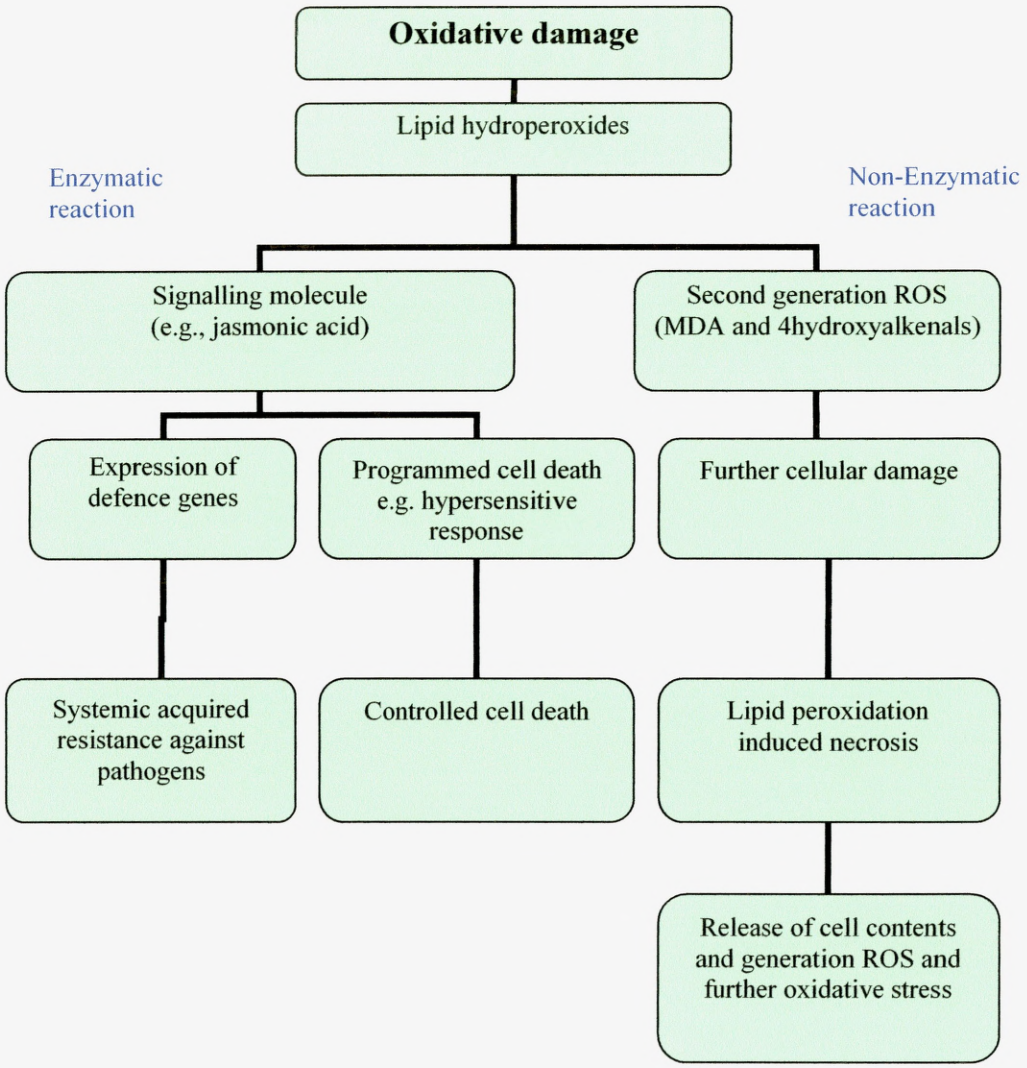


Figure 1.17: Flow chart to summarise the various pathways involving lipid hydroperoxides in cells. On one side of the diagram there are the enzymatic reactions of lipid hydroperoxide species, where they are enzymatically converted to signal molecules such as jasmonic acid and where they can induce programmed cell death and the associated expression of defence genes. The non-enzymatic pathway leads to the production of secondary lipid peroxidation products, which lead to further ROS reactions, causing considerable damage to essential biomolecules.

1.10.3 DNA damage caused by ROS

Several ROS are active electrophiles and they can form covalent bonds with potential nucleophiles such as sulphydryl groups, amino groups and DNA bases (Witz, 1989). Superoxide radicals and hydrogen peroxide are not known to have any direct

detrimental effects on DNA (Halliwell and Gutteridge, 1988), however hydrogen peroxide can undergo, in the presence of metal ions, the Fenton reaction, which converts hydrogen peroxide to the highly reactive hydroxyl radical. The hydroxyl radical ($HO^{\bullet}$) undergoes a one-electron oxidation and can induce DNA strand breaks, DNA-protein cross links and base lesions (Cadet *et al.*, 1995). The hydroxyl radical is one of the few ROS that can react with all the DNA bases and its attached sugar moieties directly (Cadet *et al.*, 1995, Halliwell, 1999). The hydroxyl radical can cause a significant amount of DNA damage; these radicals are capable of directly reacting with DNA bases, giving rise to single point mutations at random positions. The hydroxyl radical is also capable of initiating lipid peroxidation, which results in an overall increase in ROS and the formation of toxic secondary products, in particular highly mutagenic MDA. Singlet oxygen is much less reactive than the hydroxyl radical and it undergoes a specific cyclo-addition with guanosine to form a compound known as 4-hydroxy-8-oxo-4,8-dihydro-2-oxyguanosine (Cadet *et al.*, 1995). Guanine is the most easily oxidised of the four DNA bases (Halliwell, 1999), singlet oxygen does not react with any other DNA bases (Cadet *et al.*, 1995).

Lipid hydroperoxides, carbonyl end products and intermediate products of lipid peroxidation are formed in close proximity to proteins where DNA is embedded or attached and are thought to cause considerable DNA damage (Yang and Schaich, 1996). Lipid hydroperoxides and carbonyl compounds are long-lived species and are capable of moving between cells and tissues. In comparison, the hydroxyl radical has a half-life of only 10^{-9} seconds while lipid hydroperoxides have a half life of up to 7 seconds (Termini, 2000) and not only react with DNA but as they are long lived they

can initiate a cascade of reactions producing more free radical species capable of further DNA damage.

Alkoxyl radicals are intermediate radicals formed during lipid peroxidation; they have a short half-life and are capable of reacting with DNA in the vicinity of its production site (Termini, 2000). Alkoxyl radicals are capable of hydrogen abstraction directly from DNA bases and from the carbohydrate backbone. The intermolecular rearrangement of alkoxyl radicals to form peroxyl radicals is very rapid; therefore alkoxyl radicals are not thought to cause a significant amount of DNA damage (Termini, 2000). Peroxyl radicals are long lived and react with the sugar moieties attached to DNA bases. These radicals are not capable of directly damaging the individual DNA bases; however the DNA damage caused has been observed quite some considerable distance from their site of production (Termini, 2000).

MDA and 4-HNE react with nucleic bases to form multiple adducts and MDA, having two functional carbonyls, (see Figure 1.15) can form adducts from both ends (Marnett, 1999). These DNA lesions caused by adducts are potentially lethal to the cell and can have two main effects: the activity of DNA polymerase can be decreased (Laval, 1995) and, secondly, the DNA repair enzymes can be inhibited (Wiseman and Halliwell, 1996), the outcome being a increase in the frequency of mutations. Mitochondria have their own set of DNA known as mtDNA, but unlike other DNA there are no histones to protect them, which means that they are very susceptible to free radical damage (Wickens, 2001). If the DNA repair enzymes and DNA replication enzymes are damaged in the mitochondria, there will be a knock-on effect on energy production and enzyme activity.

1.10.3.1 DNA methylation

DNA methylation can have implications on gene expression, which may have consequences on phenotype, cellular growth and development. In combination with studies of oxidative stress DNA methylation may also be an effective marker of *in vitro* ageing, stress and neoplasia and for this reason formed an investigative part of this study. Moreover, DNA methylation has also been implicated in tissue culture recalcitrance in certain species which also demonstrated oxidative stress pathology. In *Vitis vinifera,* a species known to be problematic in *in vitro* tissue culture, the DNA methylation status was investigated to determine whether methylation changes were implicated in recalcitrance and *in vitro* ageing (Harding *et al.*, 1996). Methylation or demethylation of DNA or RNA causes a change in protein synthesis, which has a direct effect on growth and development. Harding *et al.,* (1996) reported that on initiation of *Vitis in vitro* cultures there were changes in methylation status. On initiation of shoot cultures there was an increase in rDNA methylation; however callus cultures showed a decrease in methylation. The data suggested that there were dynamic changes taking place in the genome during the initiation of *in vitro* tissue culture, which may be contributing to recalcitrance in this particular species.

DNA methylation is the addition of a methyl group (CH_3) to the DNA base cytosine (see Figure 1.18), which appears to be the only DNA base that undergoes such methylation. The reaction involves flipping the base to be methylated out of the double helix; a methyl group is then transferred from S-adenosylmethionine (SAM) enzymatically through the action of methyl transferases (Pufulete, 2001).

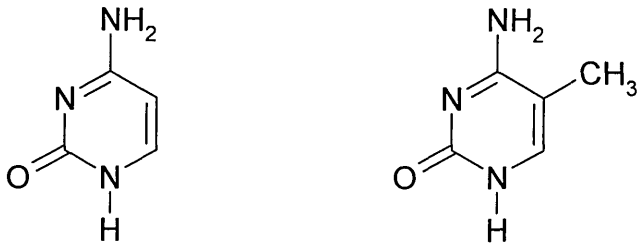


Figure 1.18: Diagram of the DNA base cytosine (left) and 5-methylcytosine (right)

DNA methylation is an epigenetic change (although the DNA base is modified the original sequence is still intact). Methylation occurs in cytosine bases that are attached to a phosphodiester, paired with a guanine, known as a CpG nucleotide (Pufulete, 2001). These CpG nucleotides are clustered in small islands in normal cells and 40% of tissue specific genes are thought to comprise of CpG islands (Pufulete, 2001). They are found at active sequences and promoter regions and are often known as "housekeeping genes" as they are actively expressed in all cells (Catania and Fairweather, 1991). In general, genes that are under methylated are considered to be active and heavily methylated are thought to be inactive (Lambé *et al.*, 1997).

Methylation has only been observed in the animal and plant kingdoms, with the percentage of methylation in animal tissues being usually between 4-6%. However, in plants the extent of methylation can be up to 30% (Gruenbaum *et al.*, 1981). The general mechanism of methylation is the same for both but there are differences in the target DNA sequences and type of methyl transfers utilised and there are more methyl transferases found in plant species, which may account for the higher extent of methylation. There are several different proposed roles for DNA methylation, including strand selection in mismatch base repair (Hare and Taylor, 1985) and recognition sites for restriction endonucleases (Riggs, 1990). DNA methylation may act as a code for specific proteins which bind to the DNA and may be responsible for

changes in chromatin structure by activation of the histones responsible for chromatin organisation (Wade *et al.*, 1999). Endogenous genes, which are original genes involved in plant cell development, tend to be under methylated. During the greening of maize leaves the encoding of photosynthetic protein was accompanied by decreased methylation (Ngernprasirtsiri *et al.,* 1989). The silencing or activation can have huge implications on gene expression, leading to decreases in protein production, cell growth, and possible cellular dysfunction.

Methylation patterns compared in normal and cancerous mammalian cell lines have been found to be very different (Pufulete, 2001). Costello *et al.* (2000) found that the extent of methylation in different tumour types was varied; they showed that several loci were heavily methylated in specific tumours. As discussed in section 1.7 in untransformed cells the oncogenes remain silenced, possibly by methylated coded sites for this gene and tumour suppression genes are un-methylated and remain active. However in tumour cells this balance is upset and the oncogenes are activated, possibly by changes in the methylation patterns (Pufulete, 2001, Costello and Plass, 2001, Costello *et al.,* 2000 and Rush and Plass, 2002). DNA mutations caused by free radical species can change DNA sequences and hence DNA methylation patterns, through un-repaired strand breakages and base deletions; which can cause changes in the activation or silencing of genes (Catania and Fairweather, 1991).

1.11 ANTIOXIDANT SYSTEMS

Due to the nature of respiration and other oxygen dependent substrate oxidations, the production of ROS is unavoidable; however systems exist in aerobic biological cells to reduce the risk of prolonged or extreme oxidative damage. Antioxidants are responsible for the detoxification and removal of ROS and the toxic by-products of ROS reactions. To recap on the main ROS formed the following equations show the main products of prooxidant reactions with oxygen (see Figure 1.19).

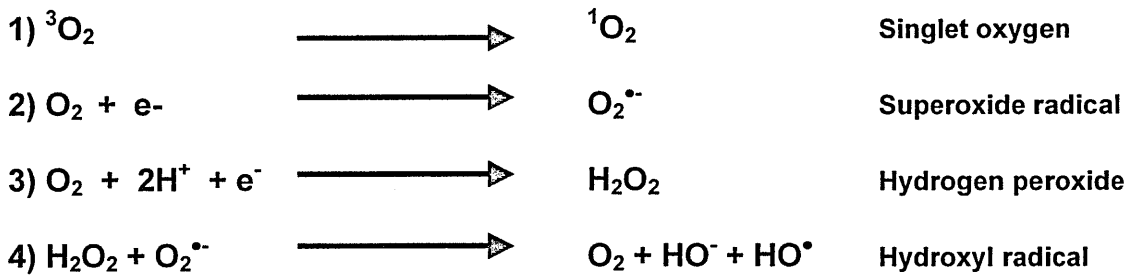


Figure 1.19: Reactions with oxygen forming pro-oxidants in biological systems: 1) Formation of singlet oxygen (lowest excited state of the dioxygen molecule), reactive towards several biomolecules. 2) The addition of an electron to molecular oxygen results in superoxide radicals. 3) The addition of an electron and protonation of molecular oxygen results in hydrogen peroxide. 4) The reaction of hydrogen peroxide with superoxide radicals leads to the formation of highly reactive hydroxyl radicals.

The definition of oxidative stress is "disturbance in the prooxidant-antioxidant balance (see Figure 1.20) in favour of the former" (Sies, 1985). Pro-oxidants are compounds or agents that are capable of forming ROS. Antioxidants fall into two categories: (a) enzyme systems such as SOD, catalase and peroxidase, which tend to be substrate specific or (b) radical scavengers such as ascorbate, glutathione and vitamin E, which react with a range of different ROS in various cellular locations.

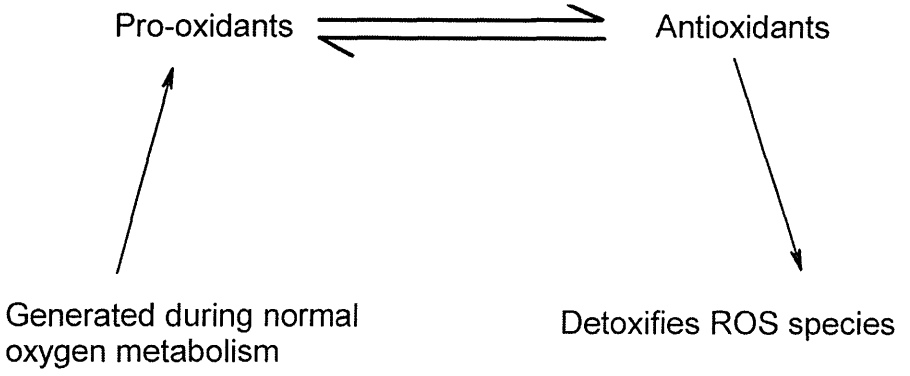


Figure 1.20: Schematic of equilibrium that exists between pro-oxidants and antioxidants. When the balance tips in favour of the pro-oxidant side, i.e. there are more ROS being formed than are being detoxified then oxidative stress occurs.

Oxidative stress induces a cellular redox imbalance; therefore to avoid oxidative stress the equilibrium must fall in favour of the antioxidants. The following section introduces and discusses the role and function of some key antioxidants, which were studied during this project. If the pro-oxidant / antioxidant balance cannot be established or regained after short-term oxidative stress, then it will result in long-term chronic oxidative stress with the most likely final outcome being cell dysfunction and death.

1.11.1 Superoxide dismutase (SOD)

Superoxide dismutases are a diverse group of metalloenzymes that catalyse the dismutation of superoxide radicals through a cyclic oxidation-reduction mechanism forming molecular oxygen and hydrogen peroxide (hydrogen peroxide is then removed through the action of catalase and peroxidase). SOD can be classified into four categories: Cu, Zn-SOD, Fe-SOD, Mn-SOD and Ni-SOD with each category distinguished by a different metal prosthetic group. Cu, Zn-SOD is the most common of the four types and is found in a wide range of animal and plant species.

The active site of the oxidised bovine SOD lies at the base of a 15Å cavity, which is formed by two of the loops in the structure which contain charged residues that facilitate the electrostatic guidance of the superoxide radical to the active site of the dismutases. Cu, Zn-SOD was the first enzyme to be detected that had a free radical species as a substrate. The activity of SOD increases as a direct response to stress (Chandru *et al.*, 2003). However den Hartog *et al.* (2003) showed that at high concentrations SOD could be toxic. When SOD is in high concentrations it is capable of oxidising biomolecules by combining with its own dismutation product hydrogen peroxide forming other ROS (den Hartog *et al.,* 2003).

SOD plays an important role in the removal of ROS and has been implicated in the prevention of several diseases, such as cancer, inflammation and helps in the fight against ageing (Halliwell and Gutteridge, 1999, Allen and Balin, 2003 and Kuratko, 1998). Pharmaceutical research based on SOD has been concentrated on development of synthetic compounds that mimic the action of SOD, which would aid the treatment of ROS-induced conditions (Naughton and Fisher, 2003). The research to date has had mixed results, although some compounds have been found to be successful in one species they may have the opposite or no effect in another, therefore much more research is needed in this area (Naughton and Fisher, 2003).

1.11.2 Catalase and peroxidases

Catalase was first discovered as a hydrogen peroxide degrading enzyme, by Loew in 1901. However it was not until 22 years later that Warburg discovered that catalase has an active centre containing iron (Zámocký and Koller, 1999). Catalase is present in almost all aerobic cells and is found in peroxisomes, which are small membrane

bound bodies that are situated near to chloroplasts in plants (Halliwell, 1981b). Catalase is comprised of four protein subunits (monomers), which form a tetramer, with an active haem centre. Catalase enzymatically decomposes hydrogen peroxide (produced from the dismutation of superoxide) to form water and molecular oxygen.

Hydrogen peroxide is thought to enter the central complex of the enzyme, where the first of two catalytic reactions take place. A proton is transferred from one oxygen to another, where the oxygen-oxygen bond polarises and elongates, causing it to eventually break and produce oxygen coordinated to the iron centre. This coordination displaces water and forms Fe (IV)=O plus a haem radical which is quickly decomposed and the intact enzyme reacts with a further hydrogen peroxide molecule, which results in another water molecule and molecular oxygen being formed (Boon *et al.*, 2001).

The whole reaction leaves the haem complex unaltered, ready for another hydrogen peroxide, but also there is no unwanted free radical intermediates produced during the reaction. The structure of catalase means that the disassociation of the subunit leads to inactivation of the enzyme. The subunits are easily disassociated by free radical species, meaning that during periods of oxidative stress the activity of this enzyme may be reduced. Habituated and aged plant cultures have been shown to have very little, if any, catalase activity throughout the cell cycle (Hagège *et al.*, 1992b and Benson 1992b).

Hydrogen peroxide is produced through two main sources, the dismutation of superoxide radicals and a product of several peroxidase reactions. Recent research, based on biochemical and genetic evidence, has suggested that hydrogen peroxide acts as a signalling molecule in plant cells (Neill *et al.,* 2002, Burdon, 1996, Kairong *et al.*, 2002, Corpas *et al.,* 2001 and Bolwell, 1999). Kairong *et al.* (1999) demonstrated that during the early stages of somatic embryogenesis hydrogen peroxide concentration was higher and there was a corresponding decrease in catalase and peroxidase activity, but after 5 days catalase and peroxidase activity increased steadily back to normal levels. The results showed that when hydrogen peroxide reached a critical concentration, somatic embryogenesis was promoted; they showed that by chemically inhibiting catalase and peroxidase activity a rapid increase in somatic embryo formation resulted (Kairong, 1999). It was also demonstrated, however, that above this critical concentration the formation of somatic embryos was significantly inhibited, showing that hydrogen peroxide can also be toxic to cells above a critical concentration.

The detoxification of hydrogen peroxide via peroxidase depends on the availability of suitable substrates such as reductants, lignin and plant phenolic precursors (Baker *et al.*, 2000). Peroxidase can also take part in some oxidase reactions that result in the formation of hydrogen peroxide. Peroxidase has many isoenzyme forms and is distributed throughout many cellular organelles, in particular, the peroxisomes. Recent studies have shown that certain cell wall-associated peroxidases are involved in cell wall fortification. Increases in the activity of these peroxidases leads to an increase in the amount of lignin in stem and leaf tissues (Faivre-Rampant *et al.*, 1998).

Root and shoot formation from plants in culture has been shown to cause immediate changes in peroxidase activity (Gaspar *et al.,* 1985 and Jouve *et al.*, 1994). Wakamatsu *et al.* (1993) demonstrated that during the initiation of carrot callus, peroxidase activity rapidly decreased immediately after excision and transfer to rooting medium. Peroxidase activity then steadily increased until it reached normal levels. Accompanying this steady increase in peroxidase was an increase in protein production which increases cell division and enzyme production, hence elevated peroxidase activity (Wakamatsu and Takahama, 1993).

The observed changes in peroxidase activity may be an important part of root / shoot induction or may be a response caused by the stress of the initial excision. What exactly causes this rapid drop in peroxidase production is not quite clear, however Jouve *et al.* (1994) showed that during this period there was an inverted increase in IAA activity. However, there were differences during the steady increase of peroxidase after the initial rapid decrease but there was not the same steady increase in IAA activity (Jouve *et al.,* 1994); instead there was a sudden drop in IAA activity, which may represent the end of a induction period (Gaspar *et al.,* 1985). It has also been suggested that this rapid decrease in peroxidase activity is caused by a masking effect (Gaspar *et al.*, 1985). Gaspar *et al.* (1985) found several compounds, such as plant phenolics and auxins that were capable of binding to peroxidases causing the enzyme to lose activity. Reduced peroxidase activity has also been linked to the loss of organogenic totipotency (Chawla, 1991 and Negruitiu *et al.*, 1979).

1.11.3 Polyamines, ethylene and a common precursor for antioxidants and pro-oxidants.

It is important to consider that many antioxidant and pro-oxidant pathways share common precursors and that peroxidases in particular can be involved in a multiplicity of inter-connected pathways that can both remove and produce ROS. Thus, low peroxidase activity has been linked to accumulation of polyamines (natural antioxidants) and is a common characteristic of habituated tissues of both plant and animal species (Gaspar, 1999b). The polyamine, aminolevulinic acid (ALA) is an intermediate compound in the biosynthesis of tetrapyrroles, such as peroxidase, catalase and chlorophylls. ALA is normally converted to tetrapyrrole compounds through the enzymatic action of ALA dehydrogenase, however habituated tissues have been shown to accumulate benzoic acid, which inhibits the activity of ALA dehydrogenase (Bisbis *et al.,* 2000), thus decreasing the production of tetrapyrroles and causing an increase in the intermediate polyamines (see Figure 1.21). The accumulation of polyamines is caused by altered regulation and control of the Shemin (takes place in the mitochondria) and Beale pathways (chloroplasts), which are the pathways that produce ALA.

Ethylene as discussed in section 1.5.6 can be detrimental to plants growth if allowed to accumulate in a closed *in vitro* environment and is associated with causing hyperdricity in certain species. However, it is important to appreciate that ethylene is a major signalling compound in plants and plays important roles in stimulating several morphological responses, and it uses free radicals in its production pathway. Ethylene (C_2H_4) is one of the simplest signalling compounds found in all higher plants. Unlike

the other plant hormones, ethylene is gaseous and therefore is not actively transported or degraded within the cell, but is free to diffuse easily through cells. Ethylene concentration in cells is therefore completely dependent on its rate of synthesis and rate of diffusion. Ethylene has been associated with the following morphological responses (Davies, 1995 and Ecker, 1995):

- Stimulating the release of dormancy
- Stimulating fruit and leaf abscission
- Stimulating of flower induction and fruit ripening
- Stimulating flower and leaf senescence

In higher plants ethylene is produced from L-methionine, and methionine is activated by ATP to form S-adenosylmethionine (SAM) through the catalytic activity of S-adenosylmethionine synthetase. Also it is important to note that SAM is a substrate precursor for DNA methylation. So, providing an interconnection between ethylene, polyamines and DNA methylation; a series of pathways that at various points involve ROS and antioxidants. Starting from SAM there are two steps leading to the biosynthesis of ethylene. The first step produces 1-aminocyclopropane-1carboxylic acid (ACC). Production of ethylene from ACC is then catalysed by ACC oxidase.

The fact that the production pathways of ethylene and polyamines share the same precursor, 5-adenosylmethione (SAM) has important implications *in vitro*. Thus, the pathway shifts towards the production of polyamines and results in a decrease in ethylene synthesis in habituated tissues. The conversion of ALA to tetrapyrroles is also blocked by the presence of benzoic acid in habituated cells and therefore the

whole chain of events appears to favour the production and resulting accumulation of polyamines. These compounds are potent free radical scavengers and their increased production may benefit the removal of ROS. However, a decrease in ethylene production will ultimately lead to a disruption in normal developmental processes, which may contribute to the habituation process and loss of totipotency (Bisbis *et al.*, 2000).

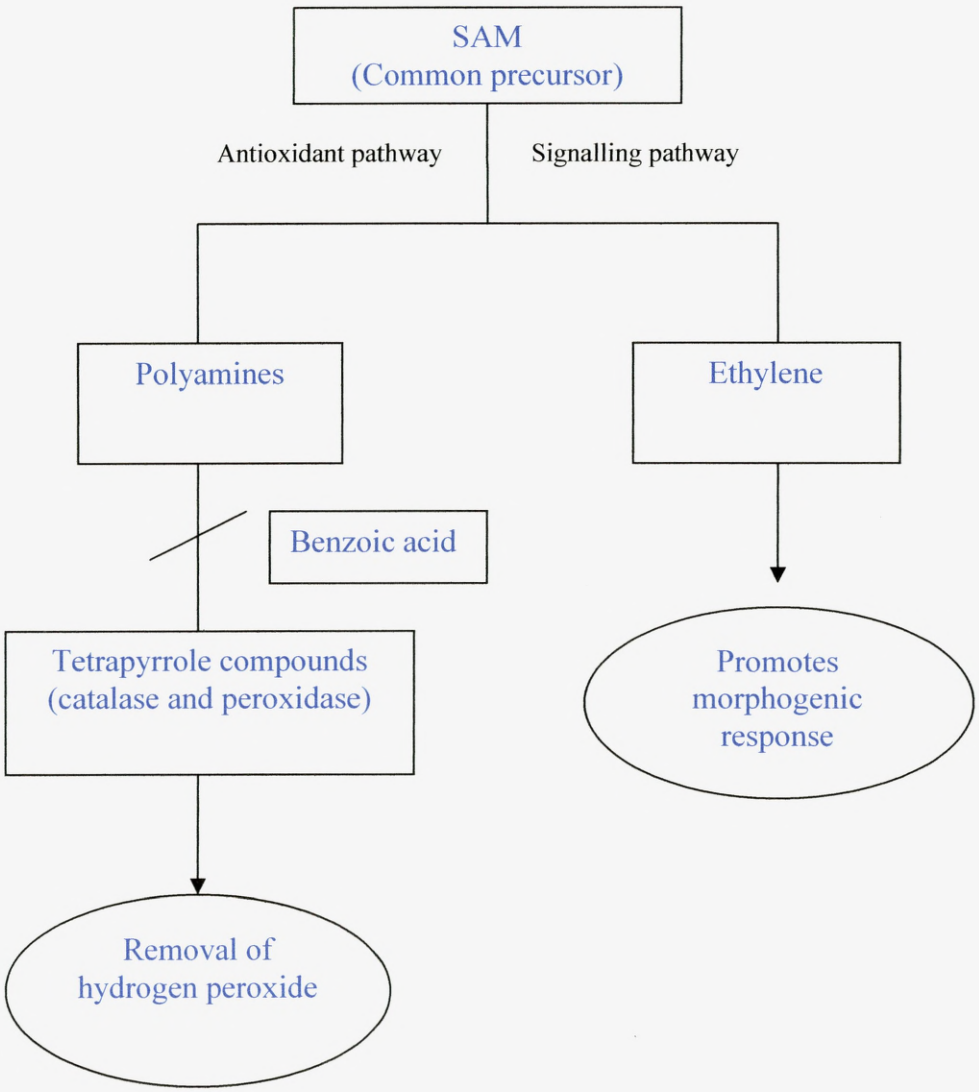


Figure 1.21: Diagram to show the antioxidant and signalling pathways that involve the common precursor SAM. The antioxidant pathway results in the production of polyamines through a reaction involving SAM; the polyamines are then converted to tetrapyrolle compounds such as catalase and peroxidase. In habituated cell lines benzoic acid is present which inhibits ALA dehydrogenase and blocks the conversion of polyamines to tetrapyrolles, which results in the accumulation of polyamines in habituated cells. The signalling pathway uses SAM to produce ethylene, which is a signalling molecule involved in several morphogenic responses.

1.11.4 Glutathione

Glutathione, a tripeptide thiol (γ-glutamyl-cystienyl-glycine) is an abundant and common low-molecular thiol, first discovered by Hopkins in 1920. Studies of the function and biochemistry of glutathione have often produced contradicting views between enzymologists and biochemists, however during the 1960s and 1970s it was demonstrated that glutathione played an enzymatic role in peptide synthesis (Cotgreave and Gerdes, 1998 and Foyer *et al.*, 2001). Today, glutathione is considered the most abundant low molecular weight sulphydryl in cells and has been recognised to play a major role in detoxification reactions. Glutathione is synthesised from glutamate, glycine and cysteine in two ATP-dependent reactions catalysed by the enzymes, glutamyl cysteine synthetase and glutathione synthetase, which are found in approximately the same concentrations in the chloroplasts and the cytosolic compartments of cells (Foyer *et al.*, 2001).

Glutathione availability is governed by three factors: the availability of cysteine (sulphur containing amino acid), the activity of the enzymes involved in the biosynthetic process (which might be inhibited by several factors including oxidative stress) and the transcriptional and translational regulation of glutamyl cysteine synthetase (which is thought to be controlled by the GSH:GSSG ratio). Glutathione is thought to be one of the most adaptable molecules in plant systems; it is required for cell growth and plays a crucial role in redox regulation and, to date, there has been no molecule identified which can mimic all of its functions.

Under non-stressed conditions the pool of glutathione is thought to be 95% in its reduced form (Carvalho and Amâncio, 2002). The initial response to oxidative stress is enhanced glutathione synthesis, which was observed in catalase-deficient mutants, where there was a dramatic increase in leaf glutathione in response to high light stress, which causes a production of excess hydrogen peroxide via photorespiration (Noctor *et al.,* 2000). Changes to the redox potential, caused by alteration of the GSH:GSSG ratio was also shown to trigger key adaptive processes such as mitosis and root developments in plants (May *et al.*, 1998).

Experiments carried out on the roots of *Arabidopsis* demonstrated that levels of reduced glutathione (GSH) were elevated in rapidly dividing cells but levels were low in quiescent (non-dividing) cells (Sanchez-Fernandez *et al.,* 1997). Belmonte *et al.* (2003) demonstrated in white spruce species that there was a critical concentration of GSH required for cell proliferation. They demonstrated that, by adding endogenous GSH, thereby increasing the GSH ratio, cell division and cell growth increased. When they added GSSG, which resulted in the decrease of the GSH ratio there was a decrease in cell division and cell growth (Belmonte *et al.,* 2003). Glutathione takes part in a number of different biochemical processes within plant cells (Foyer *et al.,* 2001), some of which are non-antioxidant reactions and some are involved in antioxidant reactions. Antioxidant reactions involving reduced glutathione (GSH) include the following:

- Detoxification of xenobiotics
- Precursor of phytochelatans
- Inducer of defence genes associated with stress resistance

- Redox regulation of the cell and acts as a buffer to maintain a stable redox potential, which aids in the protection of enzymes against deactivation (through sulphydryl oxidation)
- Re-reduction of ascorbate in the Halliwell-Asada cycle
- Direct detoxification of singlet oxygen, superoxide, hydroxyl radicals and hydrogen peroxide

As glutathione is the main source of reduced sulphur in cells and sulphur is very important in plant nutrition, it is essential that it can be transported to different cell compartments; this is achieved through plasma membrane transport systems. In maize scutella seedlings, transport of glutathione was observed in the direction of the developing roots and shoots (Rauser, 1991) and in ripening grapes the concentration of leaf glutathione sharply decreased, suggesting it was being transported to aid in the ripening process (Adams and Liyanage, 1993). Glutathione synthesis occurs in the chloroplasts and the cytosol; glutathione produced in the cytosol can be readily transported into the chloroplasts if required. The ratio of glutathione in the chloroplasts and cytosol is normally about 10:1 (GSH:GSSG) and the ratio in the apoplast is much lower at 1:1 (GSH:GSSG), suggesting that glutathione may be involved in more reactions in the chloroplast and cytosol and the reactions that take place in these particular cellular compartments require a high concentration gradient.

Chloroplasts are extremely important in plants as they are the main site of energy production and they house all the apparatus required to carry out photosynthesis. However chloroplasts are also the primary site of ROS production in plants, caused by electrons leaking from the electron transport chain, therefore the immediate removal

of ROS in the chloroplasts is essential to prevent cascading ROS reactions. The large ratio of reduced glutathione to oxidised glutathione between cell compartments allows a sufficient gradient difference to facilitate easy transport between cell compartments and rapid detoxification of ROS. When glutathione is transported against a gradient, energy is required and this is provided by ATP hydrolysis (Foyer *et al.,* 2001). Maintaining the glutathione concentration gradient is largely dependent on the rate of depletion and degradation versus the rate of biosynthesis and recycling, which is ultimately dependent on the activity of all the enzymes involved in these processes and the availability of the substrate cysteine. The concentration gradient is also dependent on the effectiveness of transport systems between cellular compartments. All of the above factors have a direct influence on the GSH: GSSG redox potential and disruption of redox regulation has serious implications on cellular reactions, which are regulated by the redox ratio.

The S-glutathionylation of proteins is essential to maintain redox equilibrium between free and protein bound glutathione. If reduced glutathione levels are decreased then the activities of important proteins can be modified (Foyer *et al.,* 2001). During periods of oxidative stress protein bound sulphydryl groups can be oxidised and so the replacement of protein bound sulphydryl is very important to maintain protein function. Membrane bound sulphydryl groups are also essential for protecting the membranes against lipid peroxidation (Cotgreave and Gerdes, 1998). The replacement of protein bound sulphydryl groups is facilitated though reduced glutathione and specific enzymes and again this reaction is under redox control and its efficiency can be affected by changes in the redox potential.

1.11.5 Ascorbate

Ascorbic acid is a water-soluble vitamin found in plants and its concentration is particularly high in citrus fruits and blackcurrant. Ascorbic acid, otherwise known as vitamin C, is found naturally in all plants and certain animals; however, in primates this compound is not produced. The role of ascorbate in primate species has not been clearly established but it is thought to be involved in the synthesis of collagen, for bone and cartilage production. Ascorbate is therefore a dietary requirement for mammalian species and if absent can lead to a condition known as scurvy. In plants, ascorbate is naturally found in the chloroplasts, cytosol, vacuoles, mitochondria and the cell wall (Anderson *et al.*, 1983), with by far the highest concentrations being in the chloroplasts (Foyer, 1993). Ascorbate takes part in a series of oxidation-reduction reactions known as the Halliwell-Asada cycle (see Figure 1.22). Ascorbate is often regarded as the main reducing substrate involved in the removal of hydrogen peroxide in plant chloroplasts.

Ascorbate peroxidase uses two molecules of ascorbate (ASC) to reduce hydrogen peroxide to water and results in the production of an intermediate product known as monohydroascorbate. This is a short-lived species that is further reduced to form dehydroascorbate (DHASC), which is a much more stable oxidised form of ascorbate. The recycling of ascorbate involves dehydroascorbate being reduced enzymatically through the action of dehydroascorbate reductase utilising glutathione (GSH) as an electron donor. The oxidised glutathione (GSSG) is then converted back to GSH through the enzymatic action of glutathione reductase utilising NADPH as a substrate (see Figure 1.22) (Noctor and Foyer, 1998 and Drazkiewicz *et al.*, 2003).

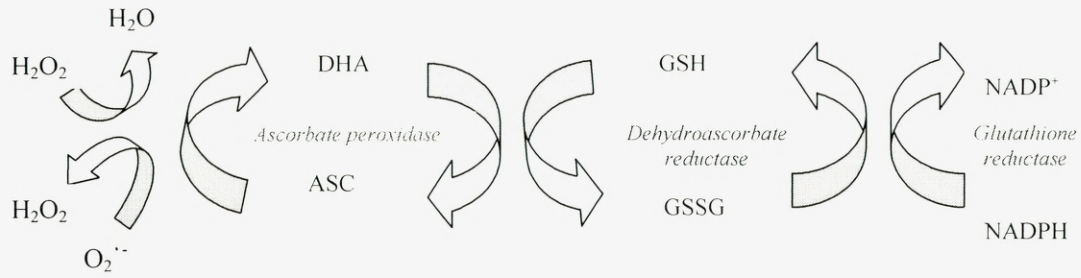


Figure 1.22: Halliwell –Asada ascorbate cycle: Ascorbate peroxidase scavenges hydrogen peroxide (H_2O_2) at the expense of ascorbate (ASC). Ascorbate is then recycled by GSH / dehydroascorbate reductase. Then the GSSG is recycled back to GSH using the NADPH-dependent enzyme glutathione reductase.

Ascorbate is considered an important antioxidant in plants and its location in the chloroplasts protects the cell from ROS produced during photosynthesis. Ascorbate is also thought to take part in a number of other cellular reactions in plants. During early growth and development ascorbate exists up to 90% in its reduced form, then gradually shifts towards its oxidised form (dehydroascorbate, DHA), where it is thought to act as a stored source of ascorbate, which if required can be re-reduced for cellular processes (Carvalho and Amâncio, 2002 and Smirnoff, 1996). Ascorbate has also been shown to undergo a rapid increase in concentration during the early stages of germination (Tommasi *et al.*, 2001); ratio changes were also seen to alter in cells progressing from G1 phase onto S phase in the cell cycle (Liso *et al.*, 1988).

Ascorbate can take part in electron transport chain reactions as it is a excellent electron donor or receiver depending whether it is in the reduced or oxidised form. Ascorbate, like glutathione is important in the redox regulation of cells and concentration gradients ensures transport between cellular compartments. This allows the redox potential to act as a signal for detoxification processes (Horemans *et al.*, 2000). As mentioned above the main detoxification process performed by reduced ascorbate is the removal of hydrogen peroxide but, like glutathione, reduced ascorbate

can also directly remove other ROS, including superoxide radicals and singlet oxygen (Smirnoff, 1996).

Ascorbate, although very capable of removing ROS is nowhere near as effective as glutathione, which may suggest that ROS removal is not its primary role. There is evidence to suggest that ascorbate plays a key detoxification role during early developmental stages; however after this its efficiency as an antioxidant decreases. Certain cell responses can only take place when there are low levels of reduced ascorbate. For example, lignin production was shown to be inhibited unless oxidised ascorbate levels were high (Horemans *et al.*, 2000), suggesting that reduced ascorbate levels must be decreased after early development for the next step to begin.

However as quoted by Halliwell "there is no such thing as a perfect antioxidant" (Halliwell and Gutteridge, 1999) and if the levels of dehydroascorbate (DHASC) exceed a certain level it can have detrimental effects on the cell. In rat liver endoplasmic reticulum oxidised ascorbate (DHASC) acts as a electron acceptor during protein thiol oxidation (Szarka *et al.*, 2002) and the accumulation of dehydroascorbate has also been associated with decreases in plant growth (de Pinto *et al.*, 2000). Without the unique recycling process of ascorbate, it is estimated that the reduced ascorbate pool would be depleted within minutes (Hossain *et al.*, 1984). As the recycling of reduced ascorbate takes place at the expense of reduced glutathione the rate of the reaction will depend on the availability of reduced glutathione and glutathione levels may be dependent on the NADPH substrate required in its recycling (see Halliwell –Asada cycle) so the whole process is finely tuned. However during prolonged oxidative stress this balance can be upset, resulting in changes in the

normal reduced and oxidised ratios, leading to adjustments to the antioxidant / pro-oxidant equilibrium.

1.11.6 Glutathione reductase

Glutathione is depleted rapidly during oxidative stress and it is imperative that the rate of glutathione synthesis exceeds the rate glutathione reduction. Reduced glutathione can also be recycled by an enzyme called glutathione reductase, which recycles oxidised glutathione back to its reduced form GSH using NADPH as an electron donor (see Figure 1.23).

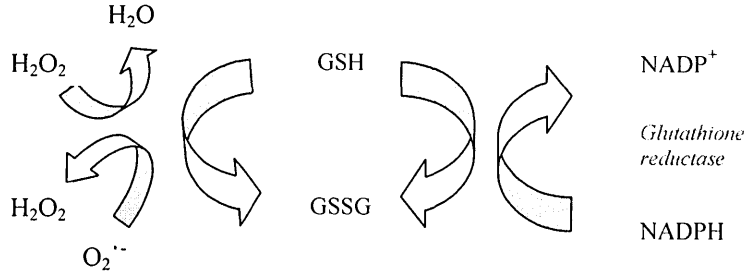


Figure 1.23: Recycling of reduced glutathione: Glutathione is capable of reducing hydrogen peroxide, leaving glutathione in its oxidised form (GSSG). Glutathione reductase is the enzyme, when coupled with the oxidation of NADPH, which re-reduces GSSG to its reduced form (GSH).

The ratio between glutathione and oxidised glutathione plays an important role in maintaining the redox potential in cellular compartments. Many cellular processes require an optimum redox range to function, therefore it is essential that a high ratio of GSH to GSSG is maintained and that the enzyme involved is carefully regulated (Foyer *et al.*, 2001). Glutathione regeneration is a very efficient process since glutathione reductase dissipates energy and aids in the adjustment of the ATP/NADPH ratio at times when carbon fixation is low. This enzyme, like glutathione levels, has been shown to be able to respond to stress rapidly. Increased

light, drought or chilling, induced an increase in the expression of glutathione reductase in chloroplasts (Foyer *et al.,* 1995). Although chloroplasts and the cytosol are the two main areas for glutathione metabolism, there was no corresponding increase in expression of glutathione reductase in the cytosol, suggesting that chloroplasts have extra antioxidant protection and can increase the production of glutathione more rapidly than the cytosol (Foyer *et al.*, 2001). GSSG, the product of GSH oxidation, can be harmful within the cell if allowed to accumulate, as is the case with the accumulation of oxidised ascorbate (DHA). Oxidised glutathione (GSSG) can inactivate enzymes by forming disulphide bonds which changes their structure and conformation and directly affects their activity (Carvalho and Amâncio, 2002).

1.11.7 Glutathione S-transferase

Plants contain GSH-dependent detoxification enzymes known as glutathione S-transferases (GST), which are ever-present multifunctional proteins. Glutathione S-transferase enzymes were first discovered in 1961 and were subsequently identified as the primary defence against reactive chemicals (Booth *et al.*, 1961). Glutathione S-transferases are comprised of two 25-27 KDa subunits, which can be made from two identical gene products or from a combination of different gene products (Edwards *et al.,* 2000). There are a number of GST super families that have been identified and are grouped depending on their primary structure, e.g. Alpha, Mu, Sigma, Kappa, Phi and Zeta (Roxas *et al.*, 1997). Plant GSTs have been described as having three major roles. Firstly to catalyse the conjugation of endogenous or xenobiotic compounds to glutathione, where they are subsequently deactivated and removed from the cell through glutathione transporter pumps (Edwards *et al.*, 2000). Secondly, they are

capable of binding phytochemicals and other important metabolites and moving them to other cellular compartments within the cell, effectively acting as a protein carrier (Edwards *et al.*, 2000). Finally GSTs have also been shown to have particularly high affinity for auxins and cytokinins (Marrs, 1996, Gonneau *et al.*, 1998), such as methyl jasmonate, salicylic acid and hydrogen peroxide suggesting that the carrier method aids in the regulation of plant hormone homeostasis.

Many types of GSTs are substrate specific (Roxas *et al.,* 1997) and an increase in expression has been previously linked with higher levels of lipid peroxidation products, such as 4-HNE, MDA, HHE and acrolein (Fukuda *et al.*, 1997). The majority of 4-HNE produced in mammalian cells is removed through glutathione conjugation and the reaction is catalysed by glutathione S-transferases (Awasthi *et al.,* 2003). There is mounting evidence to suggest that 4-HNE is associated with stress-mediated apoptosis signalling, therefore it is essential that 4-HNE is removed rapidly from the cells to prevent the initiation of programmed cell death responses (Awasthi, 2003; Awasthi, 2004 and Uchida, 1999). Studies carried out by Fukuda *et al.* (1997) demonstrated that the reaction of specific GSTs with α-β unsaturated aldehydes yielded extremely stable adducts. The structure of α-β unsaturated aldehydes, with their conjugated double bond, adds to their stability and, in particular, 4-HNE showed the highest affinity for GST conjugation (Fukuda *et al.*, 1997).

The efficient removal of the 4-HNE conjugates (GS-HNE) is essential as their accumulation can be toxic to the cell and conjugates of this nature may its-self cause enzyme deactivation and thus the product of this enzymatic reaction can lead to its own demise if not carefully regulated. The glutathione transporter pumps located in

the membranes pump GS-HNE conjugates out of the cell where they can be detoxified. The effectiveness of the glutathione pumps is regulated by the ratio of reduced glutathione to oxidised glutathione, which is very much dependent on the availability of glutathione. This tightly regulated process can be affected by oxidative stress and if the glutathione transport pumps are impaired then it could potentially lead to conjugate accumulation.

1.12 Rationale for the study

The question posed during this study is whether ROS and hence oxidative stress play a role in habituation, plant cancer neoplastic progression and *in vitro* ageing? In doing so however, it is very important to "look at the bigger picture" with respect to the complexity of interactions between different pro- and antioxidants and the fact that effecting one a will cause a dynamic that places more "pressure" on another. In doing so antioxidants can become pro-oxidants and *vice versa*. Therefore it is crucial to consider the effects the change in activity in one antioxidant has on other antioxidant system. The following flow diagram (see Figure 1.24) gives a general overview of the antioxidant and pro-oxidant pathways, which underpins the rationale for designing this thesis study. This "Road Map" highlights the route from the initial metabolic disturbance to the potential damage caused if the antioxidant pathways fail. The study will therefore employ "markers" of pro- and antioxidant pathways to profile the oxidative status of neoplastic and *in vitro* aged plant tissue cultures, with a view to elucidating the complexities of tissue culture recalcitrance and exploring the free radical basis of plant cancer *in vitro*.

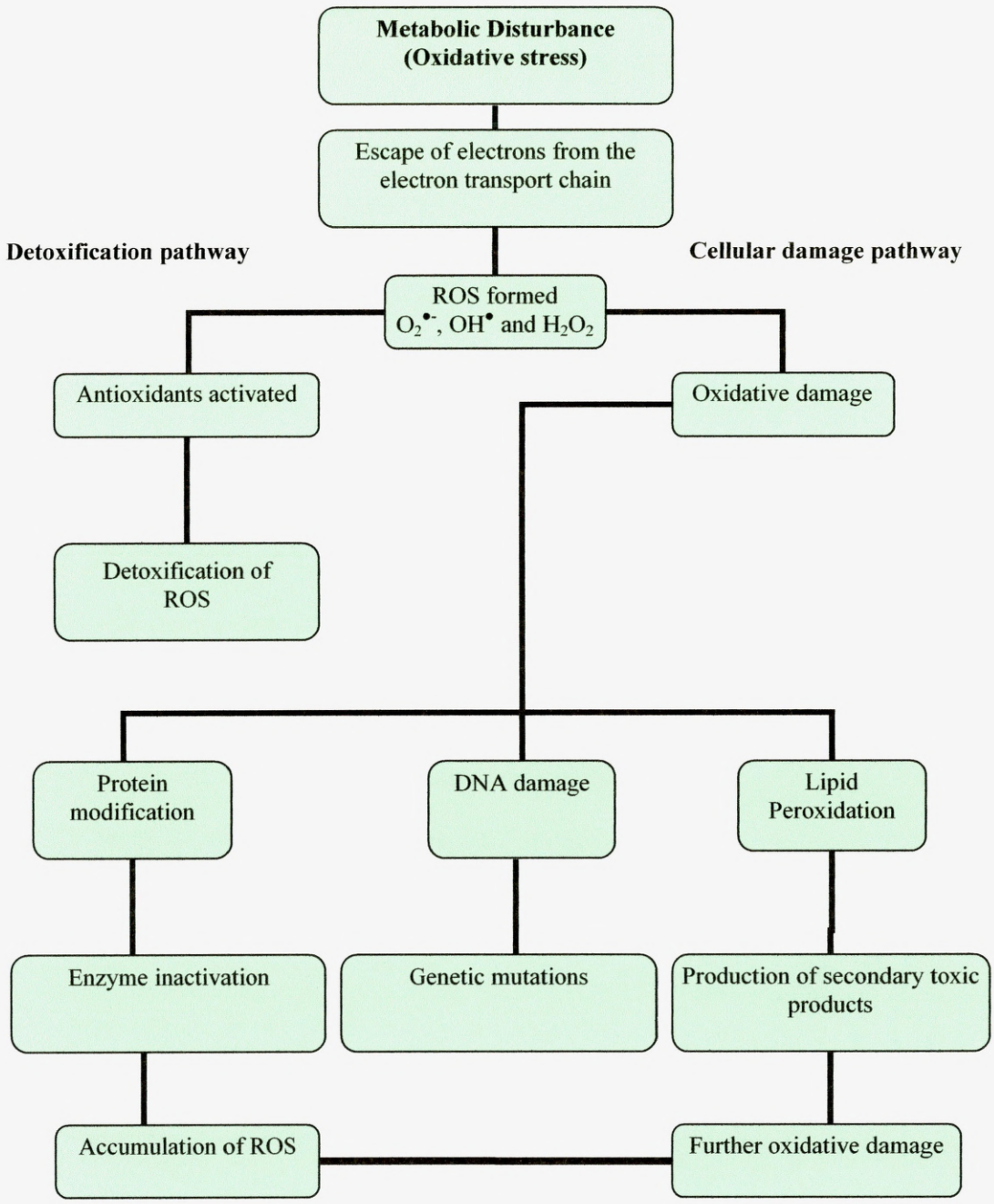


Figure 1.24: Schematic overview of oxidative stress. The process starts with a metabolic disturbance, which can result in electrons being leaked from the electron transport chain. This causes the formation of superoxide radicals and subsequent hydrogen peroxide and hydroxyl radical formation can follow. The LHS shows that the ROS are removed by the relevant antioxidant where it is detoxified and causes no further damage. On the RHS oxidative damage occurs as a result of failure of the antioxidants to remove the ROS, which can then interact with DNA causing direct mutations and can lead to several abnormalities. Lipid peroxidation can also be initiated and can produce highly toxic secondary products, which can cause further membrane damage, enzyme deactivation and DNA mutation. Protein modification can also occur, which has implications on enzyme activity and can lead to irregularities.

2.0 CHAPTER TWO: MATERIALS AND METHODS

2.1 PLANT CULTURES

- *B. vulgaris*

In October 2000, four *B. vulgaris* cell lines were kindly donated by Professor Thomas Gaspar for study in this research programme. These unique cell lines are extremely rare and were initiated in 1979 from the same original explant (De Greef and Jacobs 1979). Three of the cell lines were habituated using a combination of cold and hormone treatment (see section 1.6.2) and each cell line is considered to be going through neoplastic progressions towards a fully cancerous state. The four cell lines have different characteristics and cells lines are designated as N (normal, non-habituated), HO (habituated organogenic) and N1 and N3 (fully habituated cell lines).

The N (normal) cell line requires plant growth regulators (PGRs) supplemented in the growth medium to sustain dedifferentiated growth (callus). The other cell lines (HO, N1 and N3) are habituated and no longer require PGRs in the medium to maintain growth and are considered to be at various stages of neoplastic progression. The HO cell line in the absence of any growth regulators forms shoot-like structures, whereas two fully habituated cell lines grow completely dedifferentiated (callus) in the absence of PGRs and are incapable of forming any organised structures.

- *G. max*

G. max cultures (SW and SG) were obtained from the John Innes Centre, Norwich. Two cell lines (taken from the same strain) have been maintained in culture for approximately ten years before this project started. The soya cell line designated as SW, is an aged white callus that has lost the ability to produce any photosynthetic pigments. The cell line designated, as SG is an aged, pigmented (green) callus line. These cell lines are not habituated but have been held *in vitro* culture conditions for a considerable period of time and are considered "aged" cultures.

2.1.1 Growth requirements

The *B. vulgaris* line N requires plant growth regulators benzylamino purine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D) included in the medium to maintain dedifferentiated growth (see Table 2.1 for PGR concentrations) but *B. vulgaris* cell lines HO, N1 and N3 do not require growth hormones in the medium. *B. vulgaris* cell lines N, N1 and N3 are sensitive to photoxidation and are therefore kept away from direct light, by placing them under a layer of muslin cloth. The *G. max* cell lines SW and SG both require plant growth regulators naphthalenacetic acid (NAA) and 2,4-D for dedifferentiated growth (see Table 2.1 for PGR concentrations). All cultures were kept in a climate-controlled growth room, at 25 ^{o}C in a 16 hr photoperiod with cool white fluorescent tubes at a photon flux density of 50μmol m^2 s^{-1}. Table 2.1 gives an overall summary of growth and light requirements for each cell line.

Culture	Cell line	Growth conditions
B. vulgaris	N	25 °C, 16 hr photoperiod, not directly under light stack
B. vulgaris	HO	25 °C, 16 hr photoperiod, directly under light stack
B. vulgaris	N1, N3	25 °C, 16 hr photoperiod, in diffused light not directly under light stack
G. max	SW	25 °C, kept in dark
G. max	SG	25 °C, 16 hr photoperiod directly under the light stack

Table 2.1: Summary of the growth requirements, in terms of light and temperature, required for the *B. vulgaris* and *G. max* cell lines

2.1.2 Routine culture media

There are three types of culture media for the above cell lines: one type for the *G. max* cell lines and two for the *B. vulgaris* cell lines. The media is prepared from five stock solutions (see Appendix 6.1), which are prepared and kept in the fridge (vitamin stock solution kept in freezer). PGRs are dissolved to the appropriate concentrations (see Appendix 6.1) and are maintained at -20 °C. For 1L of *B. vulgaris* medium the stock solutions were mixed as follows: A (100mL), B (1mL), C (20mL), D (10mL) and E (5mL) were added to 300mL of distilled water. Sucrose was then added while stirring (30g/L) and once dissolved the pH was adjusted to 6.0 using 0.5M NaOH and then the solution was made up to a total volume of 1L. For *G. max* media (1L) stock solutions A, B and C (50mL) were added to 300mL of distilled water and stirred and sucrose 30g/L was added and stirred until dissolved. The pH was then adjusted to 5.8 using 0.5M NaOH and then made up to 1L with distilled water. The prepared medium solutions were dispensed into autoclave bottles and the plant agar was added (7g/L). The media was then autoclaved at 121 °C for 20min. The hormones were filter-

sterilised and added to media after autoclaving, as benzylamino purine (BAP) is heat sensitive (see Appendix 6.1 for further details of media components).

2.1.3 Culture maintenance

In vitro tissue cultures require routine sub-culturing to keep them supplied with sufficient nutrients and the required PGRs in the media for continuous and healthy growth. Table 2.2 shows the sub-culture cycles, medium type and PGRs added to the medium for all cell lines used during this current study.

Culture	Cell line	Culture cycle	Medium	Additives
B. vulgaris	N	4 weeks	PG+	0.1mg/mL BAP 0.1mg/mL 2,4-D
B. vulgaris	HO,	4 weeks	PG	None
B. vulgaris	N1, N3	2 weeks	PG	None
G. max	SW, SG	4 weeks	S+	1mg/mL BAP 2mg/mL NAA

Table 2.2: Sub-culture cycles and media composition, including media type and concentration of the plant growth regulators required for *B. vulgaris* and *G. max* cell lines

All cell lines were sub-cultured under strict sterile conditions using a laminar flow bench and all instruments were sterilised with an alcohol dip and a burner. When sub-culturing the fully habituated cell lines (N1 and N3) the lights in the laminar flow bench were switched off to reduce the light intensity. All callus cell lines were transferred onto sterile Petri plates at the time intervals indicated in table 2.2. During sub-culture only new growth from the top of the callus was transferred leaving any brown decaying material behind. In the HO cell line only healthy growing shoots

were transferred (transfer of shoot clumps was more successful than individual shoots).

2.2 CULTURE GROWTH AND VIABILITY MEASUREMENT

2.2.1 Growth analysis

Increase in fresh weight was measured throughout the normal sub-culture period to determine the growth characteristics of each cell line. Callus (100mg) was sub-cultured onto a small Petri dish (5cm diameter) and three replicates were taken from each plate and three plates were measured (nine replicates in total). For each type of callus, the same batch of medium was used throughout. Change in fresh weight was measured in intervals of three days, throughout the entire sub-culture period using aseptic techniques. Control plates were also run to determine whether the constant handling of the material had any effect on growth. The fresh weight was plotted against the number of days to identify the lag, growth and stationary phases of the growth cycle for each cell line.

2.2.2 Microscopic analysis using a light microscope (100 X LM)

Basic structure including cellular shape, membrane integrity and cellular debris levels were examined using a light microscope fitted with up to 100 times magnification lens. Slides were prepared using liquid medium for a cell suspension using the same protocol as the routine sub-culture medium, except the agar powder was omitted in order to prevent solidification.

2.2.3 Viability testing using fluorescein diacetate (FDA)

Fluorescein diacetate (FDA) is a dye, which identifies viable cells by fluorescing yellow/ green under UV illumination. Active esterase in viable cells cleaves the stain molecule causing the intense fluorescence. Non-viable cells either appear colourless or red / orange if chlorophyll pigmentation is present (Harding and Benson, 1995b). A stock solution of FDA was prepared as follows: 0.1% (w/v) in 100% acetone or methanol was freshly prepared and stored in the refrigerator. The stain is diluted to 1µl per ml with the appropriate culture media (liquid media for each type of cultures were prepared in the same way minus the agar). A few drops of the diluted stain was added to the cells on a clean microscope slide and observed under UV illumination. The auto fluorescence of the stained cells, allows a more detailed examination of cell morphology, therefore the stain was predominantly used for examining morphology rather than measuring viability. The stain remains active for only 5-10 min therefore immediate viewing was critical.

2.3 BIOCHEMICAL METHODOLOGIES

2.3.1 General cell extraction procedure

This cell extraction method uses a homogenisation technique with the aid of liquid nitrogen; fresh weight material was placed in a chilled mortar and homogenised using a pestle with three applications of liquid nitrogen (extraction was always carried out immediately after sample collection). Once a fine powder was obtained the

appropriate buffer (as described for each individual assay) was added, thawed over ice and samples were then either frozen at -20 °C or used immediately in the assay.

- Standard phosphate buffer

Phosphate buffer was used in many of the biochemical assays and the following describes how basic phosphate buffer (0.5M) is prepared and then each assay describes the exact pH and additional substrates required for each experiment.

Phosphate buffer (0.5M)

A KH_2PO_4 3.40g in 500mL d.H_20

B K_2HPO_4 4.36g in 500mL d.H_2O

A is slowly added to B whilst stirring and the pH is adjusted to the required pH using acid or base depending pH required for assay.

2.3.2 Antioxidant assays

The activity of antioxidant enzymes superoxide dismutase, catalase, peroxidase, glutathione reductase and glutathione S-transferase were measured and results were expressed as enzyme activity per mg of protein per gram of fresh weight. Levels of glutathione, ascorbate and protein bound and non-protein bound sulphydryl groups were also measured and their levels expressed per gram of fresh weight material.

2.3.2.1 Protein extraction

The general extraction procedure described in section 2.3.1 was applied to extract the proteins from the cells. Phosphate buffer (0.5M) was prepared to pH 7.0 by adding solution A to solution B (see section 2.3.1), the final volume of buffer was noted and the following salts were added to aid in the stabilisation of the proteins in the buffer solution.

$CaCl_2$	1mM
KCl	1mM
(Na_2) EDTA	1mM

Fresh weight material (200mg) was extracted into phosphate buffer (1mL) using three applications of liquid nitrogen. Once melted, the solution (1mL) was transferred to fresh Eppendorf tubes and was left to stand on ice for 20 min with intermediate (5min intervals) vortex mixing. The homogenate solution was then centrifuged at 13,000rpm at 5 ^{o}C (inside a cold room). The supernatant aliquots (1mL) were transferred to clean Eppendorfs and stored at –20 ^{o}C until required (protein extracts were stored for a maximum of 12 weeks).

Soluble protein content was determined for the six cell lines using the Bradford Coomassie dye assay (Bradford, 1976). This spectrophotometric assay employs routine sampling techniques and the assay was performed on the protein extracts (see section 2.3.2.1). The binding of the Coomassie dye to protein causes a change in the maximal absorbance from 465nm to 595nm. This assay is highly reproducible and the dye binding process takes approximately 2 min and the product is stable for up to two

hours. Coomassie Brilliant Blue G-250 (Sigma), (100mg) was dissolved in 95% ethanol (50mL, v/v), added to 85% phosphoric acid (100mL) and made up to 1L with d.H_2O. The reagent can be stored at room temperature in foil wrapped bottles for up to one week.

The protein calibration curve was prepared from ampoules containing 2mg/mL of the protein bovine serum albumin (BSA) in the range of 50μg to 2000μg/mL. The protein standard solution (100μL) was then transferred to a 10mL test-tube and Coomassie reagent (5mL) was added and the solution was mixed using a vortex mixer. The samples were allowed to stand for two minutes at room temperature before the absorbance was measured at 595nm using a spectrophotometer. The same was undertaken for the samples using 100μL of the protein extract and the concentration of protein calculated from the standard curve. The samples were all blanked against Coomassie reagent (5mL) and 100μL of protein extraction buffer.

2.3.2.2 Superoxide dismutase

Superoxide dismutase (SOD) activity was determined spectrophotometrically by monitoring the inhibition of the reduction of nitrotetrazolium blue (NBT) at 25 °C by superoxide radicals, which are produced by light-mediated generation from riboflavin and methionine (Beauchamp and Fridovich, 1971). This reaction requires a reaction platform with four one hundred watt standard light bulbs attached, to facilitate the light mediated reaction.

Stock solutions required:

A*	Riboflavin	4mg into 100mL d.H_2O
B*	NBT	40.9mg into 50mL d.H_2O
C*	Methionine	0.746g into 100mL d.H_2O
D	Wings buffer	0.5M phosphate buffer pH 7.8 then EDTA (6.72mg) was added to 200mL

*Solutions A, B and C are light sensitive so were stored in foil wrapped bottles.

The final working buffer (also light sensitive) was made from the above solutions as follows:

A	30mL
B	10mL
C	15mL
D	100mL

The final working buffer (2.98mL) was added to a 10mL test-tube and the protein extract (20μL) or standard was added (tubes should be protected from natural light using foil to stop any light-mediated reaction occurring). Control tubes and standards were randomly distributed within a rack and all the tubes were exposed to four 100W bulbs for 20min (the tubes were positioned approximately 0.5m away from the light source). The reaction was stopped by covering the tubes completely in foil and then the absorbance was measured at 570nm immediately. A standard curve was prepared using purified SOD enzyme (Sigma) in the range 30-300U/mL. Standards were diluted in Wings buffer (solution D), which was also used as a blank in the spectrophotometer.

2.3.2.3 Catalase

Catalase is involved in the direct removal of hydrogen peroxide and can be measured using a spectrophotometric assay monitoring the decrease of H_2O_2 at 240nm (Aebi, 1983). The method uses the soluble protein extracts obtained from the protein extraction (section 2.3.2.1) and enzyme activity is expressed per milligram of protein. The phosphate buffer used is the same as in the protein extraction buffer (pH = 7.0) without the additional salts (see section 2.3.1) but in this case 3% H_2O_2 (v/v) was added to 100mL of buffer prior to the assay. The activated buffer (950µL) was added to a quartz cuvette followed by the protein extract (50µL) and the decrease in absorbance at 240nm was recorded every 10 seconds for a total of 1 min. Samples were measured against a standard curve prepared in the same phosphate buffer from the stock catalase enzyme (Sigma) in the range 28-280U/mL. Specific enzyme activity was expressed per mg of soluble protein.

2.3.2.4 Peroxidase

Peroxidase activity in protein extracts was measured spectrophotometrically, by monitoring the formation of oxidation product guaiacol by guaiacol specific peroxidase at 470nm (Castillo, 1983). The phosphate buffer was prepared as in section 2.3.1 and the pH was set to pH 6.1.

Stock solutions required:

A	Guaiacol	16mM	179µL in 100mL phosphate buffer
B	H_2O_2 (3%,v/v)	2mM	20.4µL in 100mL phosphate buffer

Activated buffer (950µL) and protein extract (50µL) were added to a plastic cuvette and the absorbance change per minute was determined by reading the absorbance every fifteen seconds at 470nm (blank against activated buffer). A standard curve was prepared from the purified horseradish peroxidase enzyme (Sigma) in the range 0.1-12 U/mL and enzyme activity is expressed per mg of soluble protein.

2.3.2.5 Reduced and oxidised glutathione

Glutathione in its oxidised and reduced forms is measured using the 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) reductase recycling assay (Anderson, 1985). The rate of formation of the coloured compound, 5-thiobis-(2-nitrobenzoic acid) (TNB) formed during the reaction, is followed spectrophotometrically at 412nm. This assay is split into two parts: the first measures the total glutathione (see equations one and two below) and in the second, total glutathione is measured by reacting directly with reduced GSH (equation one) and also by recycling GSSG, in the presence of glutathione reductase and NADPH to give GSH (equation two), which then takes part in the main reaction. When GSSG recycles two molecules of GSH are produced, therefore total glutathione can be considered as GSH + ½ GSSG.

$$2\ GSH + DTNB \longrightarrow GSSG + 2\ TNB \qquad (1)$$

$$GSSH + NADPH + H^{+} \longrightarrow 2\ GSH + NADP^{+} \qquad (2)$$

Since a fresh extraction is required for this assay callus (50mg) was extracted into 5% (w/v) sulphosalicyclic acid (1mL) using three applications of liquid nitrogen (see section 2.3.1). Once thawed, aliquots (1mL) were transferred to clean Eppendorfs,

vortexed and incubated on ice for 10min. The samples were then centrifuged and the supernatant was transferred to clean Eppendorfs and stored at –20 °C until required (glutathione assays were carried out within two weeks of extraction). Assays for reduced and oxidised glutathione were performed on the same extract with the addition of the GSH inhibitor, 2-vinylpyridine, for the GSSG assay.

Stock solutions required:

A	NaH_2PO_4	0.143M	22.3g in 1L d. H2O pH 7.5*
B	DTNB	6mM	0.238g in 100mL of A
C	β-NADPH	0.248mg/mL	24.8mg in 100mL of A
D	GR reductase	138.4U/mL	100ul in 900µL of A

*A (phosphate buffer) was made up in 800mL of water then the pH was adjusted to pH 7.5 using 1M NaOH, before diluting to a final volume of 1L using dH_2O.

- Glutathione (GSH)

Cuvettes (1mL) were charged with 700µL of C and 100µL of B and 180µL of d.H_2O and then mixed. The sample or standard (10µL) was added to the mixture and the cuvette was placed inside the spectrophotometer. To start the assay, 10µL of D was added and measurements were recorded at 412nm every 5 seconds to establish the absorbance change per min. A standard curve was prepared using GSH (Sigma) in the range 0-100µM. The spectrometer was blanked using all components of the mixture except the sample component, which was replaced with distilled water.

- Oxidised glutathione (GSSG)

The samples were pre-treated with 2-vinylpyridine (2-VP) to bind existing GSH and then triethanolamine to prevent SH oxidation. 2-VP (2μL) was added down the side of a 1.5mL Eppendorf containing 100μL of the SSA extract and then triethanolamine (6μL) was added. The solution was then vigorously mixed by vortex and then allowed to incubate at room temperature for 60min to allow re-reduction of all the oxidised glutathione (GSSG) back to reduced glutathione (GSH). Then the assay was carried out in exactly the same way as the reduced glutathione assay and the amount of GSSG was calculated from its GSH equivalents.

2.3.2.6 Reduced and oxidised ascorbate

The primary role of ascorbate is the detoxification of hydrogen peroxide; however it also acts as a co-factor in many other reactions. The regeneration of ascorbate is essential to maintain its redox potential and efficiency as an antioxidant. Ascorbate (ASC) and dehydroascorbate (DHASC) concentrations were determined using a method based on the reduction of Fe^{3+} to Fe^{2+} in an acidic solution. On reaction with bathophenanthroline the Fe^{2+} ion forms a red coloured complex, which has a maximum absorption at 534nm. Total ascorbate was determined by the reduction of dehydroascorbate to ascorbate using dithiothreitol. The dehydroascorbate concentration was determined from the difference between the total ascorbate and reduced ascorbate concentrations (Nakagawa and Sagisaka, 1984).

Stock solutions:

A	Trichloroacetic acid	5% (w/v) in d.H_2O
B	Absolute ethanol	99% (v/v)
C	Dithiothreitol	0.06% (w/v) in ethanol
D	Na_2HPO_4; NaOH	0.2M; 1.2M in d.H_2O
E	N-ethylmaleimide	0.24% (w/v) in ethanol
F	Trichloroacetic acid	20% (w/v) in d.H_2O
G	Phosphoric acid	0.4% in ethanol
H	Bathophenanthroline	0.5% (w/v) in ethanol
I	$FeCl_3$	0.03% (w/v) in ethanol

Fresh weight material (200mg) was extracted into chilled solution A (1.5mL), using 3 applications of liquid nitrogen (see section 2.3.1). Once thawed, aliquots (1mL) were removed and placed in fresh Eppendorf tubes and centrifuged at 14,000rpm for 10 min and the supernatant was then transferred to clean Eppendorfs and used directly in the assay.

- Total ascorbate (ASC)

The sample or standard (0.3mL) was placed in a clean dry test tube and 5% (w/v) trichloroacetic acid, (0.3mL) and absolute ethanol (0.3mL) were added. Then 0.24% (w/v) N-ethylmaleimide (0.15mL) and 20% (w/v) trichloroacetic acid (0.3mL) were added to adjust the pH to between pH 1-2, and the mixture was gently mixed. The colour was developed by the addition of 0.4% (v/v) phosphoric acid (0.15mL), 0.5% (w/v) bathophenanthroline (0.3mL) and 0.03% (w/v) $FeCl_3$ (0.15mL). The mixture was then vortexed and incubated at 30 °C for 90min and the tubes were removed from the water bath and the absorbance was read at 534nm. A standard curve was prepared

using ascorbic acid (Sigma) in the range 0-10μg/mL to determine the concentration of the samples. The spectrometer was blanked against all the components of the mixture except the sample, which was replaced with distilled water.

- Dehydroascorbate (DHASC)

To calculate the concentration of dehydroascorbate, the total ascorbate (dehydroascorbate + ascorbate) was determined. Dehydroascorbate must first be converted back to its reduced form (ascorbate), using dithiothreitol (DTT). The sample or standard (0.3mL) was placed in a clean dry test tube and 0.06% (w/v) dithiothreitol (0.15mL) and 99% (w/v) absolute ethanol (0.15mL) was added and gently mixed and was left to incubate at room temperature for 10min. The assay was then repeated, as in the ascorbate assay, starting with the addition of 0.24% (w/v) N-ethylmaleimide (0.15mL) and 20% (w/v) trichloroacetic acid (0.15mL). The colour was developed by the addition of 0.4% (w/v) phosphoric acid (0.15mL), 0.5% bathophenanthroline (0.3mL) and 0.03% (w/v) $FeCl_3$ (0.15mL). The mixture was then vortexed and incubated at 30 ^{o}C for 90min. After incubation, the tubes were removed from the water bath and the absorbance read at 534nm. A standard curve in the range 0-10μg/mL was used to determine the concentration of the samples and the same reference was used to blank the spectrophotometer.

2.3.2.7 Glutathione reductase

Glutathione reductase activity was determined spectrophotometrically using the Goldberg method (Goldberg and Spooner, 1983). A decrease in absorbance due to the

oxidation of nicotinamide-adenine dinucleotide diphosphate (NADPH) at wavelength 334nm was measured. Glutathione reductase, catalyses the splitting of oxidised glutathione (GSSG) back to its reduced form (GSH) with the co-oxidation of NADPH to $NADP^+$. Glutathione reductase is not directly involved in reactions with reactive oxygen species however; it plays an important role in recycling glutathione. Samples were taken from the protein extract (see section 2.3.2.1).

Stock solutions required:

A	KH_2PO_4	0.12M/L	16.33g in 1L d.H_2O *
B	EDTA-Na_2H_2O	15mM/L	0.56g in 100mL d.H_2O
C	GSSG	65.3mM/L	40mg in 1mL d.H_2O
D	β-NADPH	9.6mM/L	8mg in 1mL 1%$NaHCO_3$ (aq)

*Phosphate buffer (solution A) was prepared by dissolving KH_2PO_4 (16.33g) in d.H_2O (800mL), the pH was then adjusted to pH 7.2 using 1M NaOH and then made up to a final volume of 1L with d.H_2O.

Cuvettes were prepared with stock solution A (2.6mL), B (0.1mL) and C (0.1mL). Finally the protein extract (0.1mL) was added and left to stand for 5 min and then solution D (50μL) was added. After gentle stirring, the absorbance was measured at 334nm every 15 sec to establish the activity change per min. A standard curve was prepared from the purified enzyme glutathione reductase (Sigma) in the range 3-30U/mL. Standards were prepared in phosphate buffer (solution A). Phosphate buffer (solution A) was also used as the reference to blank the spectrophotometer.

2.3.2.8 Glutathione-S-transferase

Glutathione S-transferase activity (GST) was measured using the artificial substrate 1-chloro-2,4-dinitrobenzene and glutathione as a co-enzyme, where two forms of the enzyme were active towards this substrate (Simons and Vander Jagt, 1977). Glutathione S-transferase is the enzyme that catalyses the conjugation of toxic compounds to glutathione to enable them to be removed from the cell via glutathione transporter pumps located in the cell membrane.

Stock solutions required:

A	KH_2PO_4	0.2M	2.72g in 100mL d. H2O
B	$NaHPO_4$	0.1M	3.12g in 200mL d. H2O
C	Glutathione	17.5mM	0.1076g in solution A (20mL)
D	1-Chloro-2,4-dinitrobenzene	0.1M	0.0203g in 100mL 100% ethanol

The final working buffer was prepared from the above stock solutions as follows: solution A (8mL), solution B (28mL) and distilled water (64mL). The pH of this solution was adjusted to pH 6.5 using 1M NaOH, and then solution D (4mL) was added.

Plastic cuvettes were prepared with the working buffer (3mL), solution C (0.5mL) was added and the mixture was allowed to equilibrate at room temperature for 2 min, then 10μL of sample or standard was added. The absorbance was then measured at 340nm every 15 seconds to establish the absorbance change per min. A standard curve was prepared from stock glutathione S-transferase enzyme (Sigma) in the range

of 14-140U/mL in working buffer and the reference was made with all components except the sample or standard, which was replaced by distilled water.

2.3.2.9 Sulphydryl groups

Sulphydryl groups can be classified as any organic molecule containing –SH groups and are classified as free sulphydryls, such as glutathione (GSH) or they can be bound to protein units. Sulphydryl levels were determined for both the total and non-protein bound groups using a method based on the reduction of 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (Chevrier, 1988). DTNB reacts with the available glutathione forming a coloured complex, which has a maximum absorbance at 412nm. The concentration of protein bound groups is calculated by subtracting the concentration of free SH groups from the total (protein bound + non protein bound) sulphydryl value.

Stock solutions:

A	DTNB	10mM	0.3963g in 100mL methanol (98%)
B	Tris HCl	1.0M	See Sigma catalogue (pH=8.2)
C	Tris HCl	0.2M	See Sigma catalogue (pH=8.5)
D	SDS	5% (w/v)	5g into 100mL d.H_2O
E	TCA	5% (w/v)	5g into 100mL d.H_2O

- Total sulphydryl groups

Fresh weight material (200mg) was extracted into extraction buffer B (2mL) using three applications of liquid nitrogen (see section 2.3.1). Once thawed, aliquots were

transferred to fresh Eppendorfs then solutions D (0.5mL) and A (0.1mL) were added and stirred gently. The mixture was maintained at room temperature for 20 min with intermittent mixing. Ice-cold methanol (3mL) was added to the mixture and centrifuged at 10,000 rpm and the supernatant was transferred to fresh Eppendorfs. The absorbance was read at 412nm and compared to that of a standard curve prepared from glutathione (Sigma) in the range of 10-100μM. Standards were prepared in the solution B and the spectrometer was blanked against solutions B (2mL), D (0.5mL) and A (0.1mL), the sample was replaced with solution B.

- Non-protein bound sulphydryl groups

Fresh weight material (200mg) was extracted into stock solution E (1.5mL) using three applications of liquid nitrogen (see section 2.3.1) and left to incubate on ice for 10 mins after thawing. The samples were then centrifuged at 13,000rpm for 10 min and the supernatant was transferred to clean Eppendorfs. Plastic cuvettes were prepared with stock solution C (3mL) and A (0.1mL). The supernatant (1.0mL) was added to the cuvette, gently mixed and allowed to stand at room temperature for 2 min. The absorbance was then measured at 412nm and was compared against the same standard curve for the total SH groups. The standards were prepared using HCl Tris buffer (solution C) and a blank was made containing all but the sample, which was replaced with solution C. Standards were prepared in solution C, using GSH (Sigma) in the range 10μM to 100μM.

2.3.3 Reactive oxygen species (ROS)

2.3.3.1 Hydrogen peroxide

Hydrogen peroxide causes widespread damage if allowed to accumulate in cells. The detection of hydrogen peroxide utilises a sensitive colorometric assay. Hydrogen peroxide is a potent oxidiser, which couples oxidatively with 4-aminoantipyrine and phenol to produce a quinoneimine dye, which has a maximum absorbance at 505nm (Packer, 1984).

Reagents required:

A	Phenol	
B	4-Aminoantipyrine	
C	Potassium phosphate buffer	(pH 6.9, 0.1M)
D	Horseradish peroxidase	(Sigma)
E	Hydrogen peroxide	30% (v/v)

Extraction Procedure:

The original protein extracts cannot be used in this assay as hydrogen peroxide is thought to decompose over time, therefore to get a more accurate result fresh protein extracts were utilised (see section 2.3.1). Fresh weight material (100mg) was extracted into phosphate buffer (0.1M, pH 6.9) using three applications of liquid nitrogen (see section 2.3.1). In this assay the phosphate buffer is a different concentration, so 0.1M A and 0.1M B were prepared first before the solutions were

mixed (see section 2.3.1). Aliquots (1mL) were placed into Eppendorf tubes and centrifuged at 14,000 at 4 °C, for 10 min. The supernatant was transferred to fresh Eppendorf tubes and stored at –20 °C (the assay was performed within twenty four hours of sample extraction).

Stock solutions:

The working solution was prepared fresh daily as follows and maintained at an ambient temperature throughout the experiment, solutions A (0.234g), B (0.1g), C (1mL) and D ($2x10^{-8}$M) are made up to 100mL in distilled water. This working reagent solution (4mL) was mixed with the sample or standard (500uL), and then made up to 10mL with distilled water. The change in absorbance at wavelength 505nm was monitored, until a constant reading was obtained and the absorbance change over the first minute was calculated. The reference sample was made up using reagent solution (4mL), and phosphate buffer (0.5mL) then made up to 10mL with distilled water. The concentration was calculated from a standard curve in the range 50-200µL/mL using a stock solution of 30% (v/v) hydrogen peroxide (Sigma).

2.3.3.2 Detection of hydroxyl radical activity

When plants undergo oxidative stress hydroxyl radicals are produced, which cannot be measured directly, without very expensive and complex methods. However in the presence of dimethyl sulphoxide (DMSO) hydroxyl radicals are "trapped" and convert DMSO into methane gas, which can be analysed using gas chromatography (Fleck *et al.*, 2000). Hydroxyl radicals ($HO^{\bullet}$) react with DMSO to form methyl radicals ($CH_3^{\bullet}$)

and the methyl radical then abstracts further hydrogen to form methane, which is detected using gas chromatography.

Stock solutions:

A	DMSO	1% (v/v) solution in d.H_2O
B	Methane	Gas standard (mix 54, containing 15ppm methane, Supelco)

Sample preparation:

Fresh medium was prepared for each cell line and 15mL vials (fitted with septa) were autoclaved, left open in the laminar flow bench to allow any volatiles accumulated from the autoclave process to escape. After 30 min the melted media was transferred into the vials (2mL per vial) using sterile pipette tips. The vials were then allowed to set and cool for 30 min prior to screwing the lids back on. The vials were left for one week before sample preparation to ensure they were free from contamination. At the time of the next routine sub-culture the vials were prepared for the GC experiment. Healthy callus (100mg) was transferred to the surface of the media in the vials. Three replicates were taken from each plate and three plates were sampled from each line, so the total number of replicates was nine. The callus in the vials were sealed and placed in the growth room, under normal growth conditions, until 24 hrs prior to mid cycle.

Twenty-four hours prior to the beginning of sampling DMSO (1%, 500μL) was filter sterilised using a 0.2μM pore filters (Whatman) and added to the surface of the media in each of the vials; filter sterilised water (500μL) was used in the control vials. The time was noted when the vials were sealed and placed in the growth room: twenty-

four hours later the vials were analysed for methane production. A sample of gas was removed from the headspace (1mL) and injected into the GC using a gas syringe. The concentration of methane was calculated from a standard curve prepared from a mixture of gas standards supplied by Supleco. After sampling, the vials were transferred to the laminar flow bench where they were opened and vented for 20 min then sealed and placed in the growth room for another twenty-four hours. Sampling continued this way for five days to get a snapshot of the average amounts of methane being produced per day in the mid cycle growth period of the cell lines.

2.3.4 Lipid peroxidation products

Lipid peroxidation is one of the major consequences of oxidative stress in plants and the products produced by lipid peroxidation are often toxic to the cell and can initiate further free radical reactions. Primary products of lipid peroxidation, lipid hydroperoxides and conjugated dienes were measured alongside the secondary products MDA, 4-hydroxynonenals and long term markers Schiff's bases. Conjugated dienes and lipid hydroperoxides can both be measured directly from the organic fraction obtained during the Schiff's base assay, therefore the Schiff's base assay is the first assay described in this section.

2.3.4.1 Schiff's bases assay

Schiff's bases are formed by the reaction of carbonyl compounds, such as malondialdehyde and 4-hydroxyalkenals with the amino side chains of a protein structure. The fluorescence emitted by these pigments is measured according to the

Lunec and Dormancy method (Lunec and Dormandy, 1979). The intensity measured is an indicator of accumulation of aldehydes as a direct result of lipid peroxidation.

Stock solutions:

A	Potassium phosphate	0.05M (pH 7.0)
B	Lipid extraction solution	2:1 Chloroform: methanol

Fresh extractions were required for this assay, the lipid extraction solution (B) was heated to 45 °C prior to the assay and the potassium phosphate buffer was prepared as in section 2.3.1; however the concentration of solution A and B was prepared to 0.05M instead of 0.5M. Solution A was added to B until pH 7.0 was achieved and then the following salts were added to a known concentration to obtain the required concentration of each salt (see below) and the final solution was chilled on ice prior to use in the assay.

KCl	0.1mM
$CaCl_2$	1.0mM
EDTA	0.001M

Fresh weight material (200mg) was extracted in potassium phosphate (solution A, 1mL) using three applications of liquid nitrogen (see section 2.3.1). Once thawed, aliquots were transferred into Eppendorfs and centrifuged at 10,000rpm at 4 °C for 10 min. The supernatant was discarded and the pellet was placed in lipid extraction solution (solution B, 6mL) and vortexed for 2 min. The solution was centrifuged for 5 min at 1000 rpm to remove protein precipitate. The supernatant was transferred to a

clean tube and deionised water (2mL) was added. The tubes were inverted slowly to separate the aqueous and solvent fractions (the tubes can be centrifuged at 1000 rpm for three minutes to aid this separation). The top aqueous layer was then removed, placed into a clean tube and any precipitate that had accumulated at the interface was removed and the lower solvent layer was transferred to a clean test tube.

At this stage, the solvent layer may be an emulsion but can be clarified by the addition of 100% methanol (200μL) prior to reading the fluorescent emission. The fluorescent emission in both the aqueous and the solvent layer was measured in the 400-500nm regions using an excitation wavelength of 360nm. The Schiff's base content was expressed as the fluorescence emission per gram of fresh weight.

2.3.4.2 Conjugated dienes

The abstraction of an aldehydic hydrogen atom from an unsaturated lipid causes bond rearrangement within the molecule, which leads to the formation of conjugated dienes, which have a maximum absorbance of 240nm. An indication of lipid hydroperoxides and conjugated dienes levels can be measured from the same organic sample extract used in the Schiff's base assay (see section 2.3.4.1) (Benson *et al.*, 1992). The samples taken from the Schiff's base organic extractions were measured in quartz cuvettes at 240nm and the chloroform/methanol (lipid extraction solution) was used as a reference to zero the spectrophotometer.

2.3.4.3 Lipid hydroperoxides

Conjugated dienes react with oxygen to form peroxyl radicals, which are capable of further hydrogen abstraction from polyunsaturated fatty acids, leading to the formation of lipid hydroperoxides, which have a maximum absorbance at 270nm (Benson *et al.,* 1992). This absorbance is characteristic of –OOH groups, which are primary products of the oxidation of unsaturated lipids. The organic samples from the Schiff's base assay can again be used and measurements were read at 270nm using quartz cuvettes and the chloroform/methanol lipid extraction solution was used as a reference to zero the spectrophotometer.

2.3.4.4 Malondialdehyde and 4-hydroxyalkenals (LPO-586 assay)

Lipid peroxidation products were measured by the formation of a coloured chromophore on the reaction of malondialdehyde or 4-hydroxyalkenals with two moles of 1-methyl-2-phenylindole (MPI) in acidic conditions. The spectrophotometric assay is based on a commercially available test kit, known as LPO-586 available from Calbiochem. A fresh extraction is required for this assay and the assay is divided into two parts; the first part measures the total concentration of malondialdehyde and 4-hydroxyalkenals and the second reaction measures the concentration of malondialdehyde only; the reaction is manipulated by changing the type of acid used for each determination (Diaz *et al.,* 1998, Erdelmeier, 1998 and Gerard-Monier *et al.,* 1998).

Stock solutions:

A	Potassium phosphate	0.5M	pH 7.0 (See 2.3.1)
B	1-Methyl-2-phenylindole	10mM	0.207g in 100ml of 99% acetonitrile
C	Hydrochloric acid	Stock	HCl (37%)
D	Methanesulphonic acid	15.4M	Sigma
E	Methanol	100%	Sigma

Prior to use in the assay the indole reagent (Solution B; 18mL) needs to be activated by the addition of methanol (6mL). This solution can be kept for up to three days at 4°C. Fresh weight material (200mg) was extracted in potassium phosphate buffer (Solution A, 1mL) using three applications of liquid nitrogen (see section 2.3.1). Once thawed the samples were vortexed for 10-15 sec. The homogenates were then centrifuged for 10min at 14,000rpm and the supernatant was transferred into fresh Eppendorfs. A water bath was preheated to 45 °C and test tubes were prepared (rinsed with dilute acid and dried thoroughly).

Indole reagent (650μL) was added to each test tube followed by the addition of either the sample or standard (200uL) and stirred gently. Then the appropriate acid (Solution C or D, 150μL) was added and incubated in the water bath for the appropriate time. For total Malondialdyhyde + 4-Hydroxyalkenals, methanesulphonic acid (Solution D) was used and incubated for 60min in the water bath. For MDA only hydrochloric acid (Solution C) was added and incubated for 45min. After the incubation period, the samples were cooled on ice for 5min to stop the reaction and the absorbance read at 586nm. Standards were prepared in distilled water, in the range of 10-100μM, using a hydrolysed solution of 4-hydroxy-2-nonenal diethyl

acetal (HNE source) and hydrolysed tetraethoxypropane (TEP), as the source of MDA, the spectrometer was blanked against distilled water. The following equation was used to calculate the concentration in the samples:

[MDA + 4 hydroxyalkenals] = (A-Ao)*5/ε

[MDA] = (A-Ao)*5/ε

A = Absorbance at 586nm for the sample

Ao = Absorbance of the blank

5 = Dilution factor

ε = Apparent molar extinction co-efficient factor (155mM/cm)

2.3.4.5 Thiobarbituric acid reactive substances (TBARS)

Malondialdehyde (MDA) and several other reactive aldehydes form coloured complexes with thiobarbituric acid (TBA), which can be detected by either spectroscopy or fluorimetry. The level of TBA reactive substances was measured using a widely used spectrophotometric assay developed by Heath and Packer (1968).

TBARS reagent:

The TBARS reagent was prepared by mixing a 0.5 % (w/v) TBA in 20 % (w/v) trichloroacetic acid (TCA) was made freshly each day and stored in the dark. TBA proved difficult to dissolve in the trichloroacetic acid solution; however by dipping

the solution beaker containing the TCA and TBA into an ultrasonic water bath greatly improved the process.

Fresh weight material (100mg) was weighed out into a centrifuge tube, distilled water (1.4mL) was added and the sample mixed. The TBA reagent (1.5mL) was added and the tubes were then placed in a boiling water bath for twenty five minutes. After this time the samples were placed on ice for five minutes to stop the reaction. The samples were centrifuged at 2,500rpm for ten minutes to remove any cell debris (optional) and the absorbance of the supernatant was measured at 532nm (specific value) and 600nm (non-specific values), the spectrometer was blanked against TBARS reagent. Standards were made up using hydrolysed tetraethoxypropane (TEP) in the range 100-10μM to ensure the assay was accurate (TEP was hydrolysed using dilute 0.01M HCl mixed gently for 1hr). As this assay is not specific to MDA, the results were expressed as a reactivity index.

2.4 MOLECULAR ANALYSIS

2.4.1 DNA analysis

2.4.1.1 DNA (CTAB) Extraction

The isolation of plant DNA is possible by using the a modified hexadecyltrimethylammonium bromide (CTAB) method (Rodgers and Bendich, 1988) and is applicable to a wide range of plant species (Harding and Benson, 1995a). The reaction involves a number of stock solutions, which are all listed in the

following tables, from the stock solutions the extraction buffers are then prepared and applied in the assay. Fresh extraction were required for this assay.

Stock solutions required:

A	EDTA	0.5M	Dissolve EDTA (186.12g) in d.H_2O (400mL) containing NaOH (10g). Adjust the pH 8.0 (1M NaOH) and make up to 500mL
B	NaCl	5M	Dissolve NaCl (145g) in d.H_2O ((500mL)
C	Tris buffer	60.55g in 500mL	Dissolve 60.55g Tris HCl in d.H_2O (400mL) and adjust the pH 8.0 using 1M HCl, then make up to 500mL with d.H_2O

The following extraction buffers are prepared from the above stock solutions:

A	Extraction Buffer	A (20mL, 20mM) B (140mL, 1.4M) C (50mL, 100mM) 1% PVP (5g, w/v) 10% CTAB (50g, w/v)	There is a specific order in which these should be mixed: PVP was dissolved d.H_2O (200mL). CTAB was added, dissolved in the salt solution (B, 140 mL). Then Tris buffer (C, 50mL) was added, and finally EDTA (A, 20mL) and then made up to 500mL with d.H_2O
B	Chloroform: isoamyl alcohol	24:1 ratio	Isoamyl alcohol (2mL) was added to chloroform (48mL)
C	10% (w/v) CTAB	B (14mL, 0.7M) 10% CTAB (10g, w/v)	Add CTAB (10g) to solution B (14mL), then make solution up to 100mL with d.H_2O

D	Precipitation solution	C (25mL, 50mM) A (10mL, 10mM) 1% CTAB (5g, w/v)	Solution C and A are mixed then CTAB was added and made up to a final volume of 500mL
E	High salt buffer	A (0.5mL, 0.5M) B (100mL, 1M) C (5mL, 10mM)	Mix solutions A, B and C and make up to a final volume of 500mL
F	Isopropanol	Assay Grade (Sigma)	Kept on ice
G	70% Ethanol	70% (v/v, d.H_2O)	Kept on ice
H	Te buffer	A (0.4mL, 1mM) C (2mL,10mM)	Solution A was added to solution C and made up to 200mL with d.H_2O

Fresh weight material (500mg) was ground to a fine powder using three applications of liquid nitrogen (see section 2.3.1) and transferred to fresh Eppendorfs. Preheated extraction buffer A (0.5mL, 65 °C) was added and the tube was mixed thoroughly, then incubated in a 65 °C water bath (30min). An equal volume of chloroform/ isoamyl alcohol B was added (0.5mL) and the tube was inverted to form an emulsion; the mixture was then centrifuged at 10,000rpm for five minutes. The aqueous phase was transferred to a fresh Eppendorf and a 1/10 volume was added of 10% CTAB salt solution C. The mixture was then extracted into an equal volume of chloroform/ isoamyl alcohol B, and then centrifuged at 10,000rpm for five minutes. The aqueous phase was transferred to a fresh Eppendorf and an equal volume of CTAB precipitation buffer D was added and mixed.

Nucleic acid was not visible at this stage so the tubes were left at room temperature, in the dark, overnight. The following day the visible nucleic acid was then centrifuged at low speed (5,000 rpm) for 5 min, the supernatant was then discarded and the pellet was re-dissolved in high salt buffer E (1-2mL, minimum amount required to dissolve

the pellet). Any remaining particulate matter was removed by centrifugation at 10,000 rpm for five minutes and the DNA solution was transferred to an Eppendorf. A volume of cold isopropanol F (0.8 of total volume) was added and the pellet was centrifuged at a low speed (5,000rpm) for five minutes. The pellet was washed with 70% ethanol (solution G) and left to air-dry (30min). The pellet was then re-dissolved in TE buffer H (500µL) and the sample was stored at -20 ^{o}C until required. The concentration of DNA and RNA was determined using a spectrophotometric procedure, which measures absorbance at 260nm (Sambrook *et al.,* 1989). The CTAB extraction method gives total RNA and DNA and the concentration of both RNA and DNA was calculated from the known fact that one optical density is equivalent to 50µg/mL of DNA or RNA. Treatment with RNase enzymatically removes RNA, leaving only DNA, this allowed the levels of RNA and DNA to be calculated in the samples.

Stock solutions for DNA and RNA assay:

A	RNase A	(Sigma, 1mg/ml)
B	Sodium acetate	3M
C	Isopropanol	Assay grade (Sigma)
D	TE buffer	(see CTAB extraction)

Total DNA (10µg) from the CTAB extraction was diluted in 500µL of TE buffer (solution D). Using this sample directly the absorbance could be read at 260nm, in a spectrometer using quartz Cuvettes with TE buffer (solution D) as a reference. RNase was prepared from stock (Sigma) to a final concentration of 10mg/mL in an

Eppendorf and heated in a water bath at 80 °C for 10 min (to denature any DNases that may be present). The RNase was then diluted to a working concentration of 1mg/mL (A) and stored in the fridge.

The CTAB extracts were then heated to 60 °C for 10 min and were flash cooled on ice and RNase A (25µL) was added and incubated at 37 °C, overnight. After incubation sodium acetate (solution B) was added (0.1 volume) giving a final concentration of 0.3M sodium acetate in the mixture, cold isopropanol C was added (0.8 volume), inverted and the tubes were stored overnight at -20 °C. The following day the samples were centrifuged at 5,000 rpm for about 10min and the supernatant was discarded. The pellet was re-suspended in 500µL of TE buffer D and the absorbance was read at 260nm to give the DNA concentration only.

2.4.1.2 HPLC Analysis of DNA bases

DNA extracted from the CTAB assay was broken down into its base components through a series of chemical treatments and the ratio of cytosine and 5-methylcytosine in the samples were compared using an HPLC method based on the Matassi method (Matassi *et al.*, 1992).

Stock solutions required:

A	$ZnSO_4$	10mM
B	Nuclease P1	200 units/mL in 30mM NaAc (Sigma)
C	Tris pH 8.3	Tris base (0.5M)
D	Alkaline phosphatase	10 units/mL in 2.5M NH_4Ac (Sigma)
E	KH_2PO_4 Buffer	4mM containing 1%MeOH (v/v)

F	50 % MeOH	HPLC grade MeOH (v/v, d.H_20)
G		HPLC grade MeOH (v/v d.H_20)
H	10% TE Buffer	See CTAB extraction (v/v, d.H_2O)

The HPLC UV detector was set to 280nm and the mobile phase was phosphate buffer (solution E), which was filtered and degassed prior to use. The flow rate was set to1mL/min and the column used was a Hypersil 5μ C18 (Phenomenex©). DNA (10μg), pretreated with RNase (see section 2.4.1.1) was made up to 100μL with TE buffer (10%) and heated (100 °C for 2 min) and was then flash cooled on ice for 5 min. Solution B (5μL) was added mixed gently then solution C was added (10μL, 2 units) and was incubated (37 °C) in a water bath for 16hrs. The following day solution E (10μL) was added to increase the pH prior to the addition of the alkaline phosphatase (solution D, 10μL); the mixture was then incubated for an additional 2hrs in the water bath (37 °C). The samples (50μL) were then centrifuged and injected straight onto the HPLC column. Standards were prepared using the purified bases cytosine and 5-methylcytosine (Sigma) and an internal control was prepared using a potato DNA sample.

2.5 STATISTICAL ANALYSIS

All data collected was inputted into Excel spreadsheets (Microsoft® Excel, 2002). Data was organised into two columns of cell line and response. The data was then transferred directly into Minitab (Version 13). Where the data series had normal distribution and equal variance (95% confidence was accepted, $P>0.05$) one way analysis of variance (ANOVA) was used to determine whether the cell lines responses varied significantly from each other. Significant, highly significant and very highly

significant were designated by $P<0.05$, <0.01 and <0.001 respectively. The Kruskall-Wallis test is a non-parametric statistical test used to compare three or more samples. It is used to test the null hypothesis that at least two or more of the samples differ from the median (if at all). It is analogous to the F-test used in analysis of variance (ANOVA), which depends on the assumption that all the populations under comparison have normal distribution and equal variance but the Kruskall-Wallis test places no such restrictions on the comparison. Thus in the case where the data did not have normal distribution or equal variance the Kruskall-Wallis test was used. Although this test is less powerful than ANOVA it is the recommended test for non-parametric data.

3.0 CHAPTER THREE: A STUDY OF *IN VITRO* HABITUATION AND NEOPLASTIC PROGRESSION IN *BETA VULGARIS*

3.1 INTRODUCTION

The four *B. vulgaris* cell lines investigated in this study are at different stages of habituation and are used to determine whether oxidative stress plays a role in habituation, and ultimately, the neoplastic progression towards cancer. By profiling the activity of antioxidants and determining the level of reactive oxygen species and lipid peroxidation products, the antioxidant potential can be determined for each line. The morphological characteristics of the four cell lines are also examined to determine if unusual phenotype and or morphological and structural changes result from or are associated with habituation and oxidative stress. The data presented were collected using a number of pro and antioxidant assays, some of which are routinely used in plants and others that were adapted for use from other disciplines specifically for use in these plant cell lines.

3.2 EXPERIMENTAL AIMS AND OBJECTIVES OF *B. VULGARIS* STUDY

1. To study the morphological characteristics of the four *B. vulgaris* cell lines.

2. To profile the activity of some of the key antioxidants in the four fully habituated and non-habituated *B. vulgaris* cell lines.

3. To profile the levels of reactive oxygen species (ROS), hydrogen peroxide, hydroxyl radicals and lipid peroxidation products in the *B. vulgaris* cell lines.

*Materials, methods and experimental designs are presented in Chapter Two – Materials and Methods, see page 112 onwards.

RESULTS AND DISCUSSION

3.3 CULTURE GROWTH AND VIABILITY MEASUREMENTS

The following section presents a morphological overview of the four *B. vulgaris* lines: normal, habituated organogenic and two fully habituated lines, allowing the profiling of the growth and morphological characteristics to be identified. Growth analysis is based on fresh weight and the morphological and cytological characteristics were examined using digital photographs followed by a more detailed examination using light microscopical observations with and without a fluororescein diacetate (FDA) vital stain.

3.3.1 *Growth analysis*

***B. vulgaris* sample**	**Percentage gain fresh weight (g) Over 30 days**
Normal (N)	833%
Habituated organogenic (HO)	380%
Habituated non-organogenic (N1)	420%
Habituated non-organogenic (N3)	270%

Table 3.1: Increase in fresh weight as a percentage over a 30-day period in *B. vulgaris* cell lines (normal), HO (habituated organogenic) and N1, N3 (fully habituated).

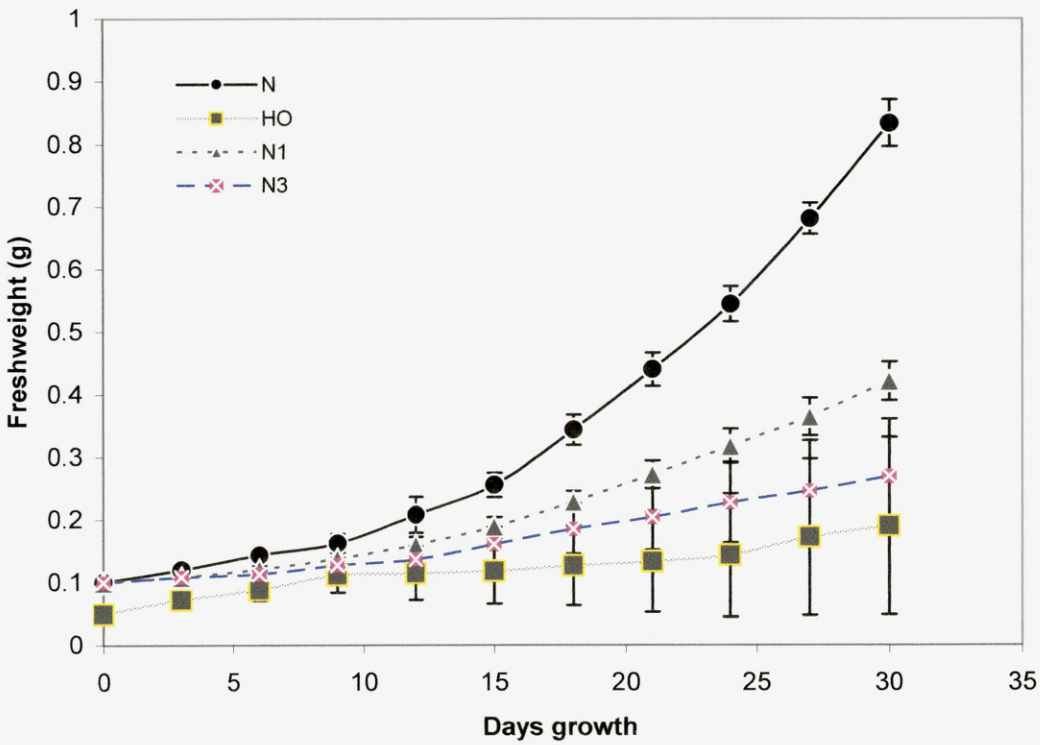
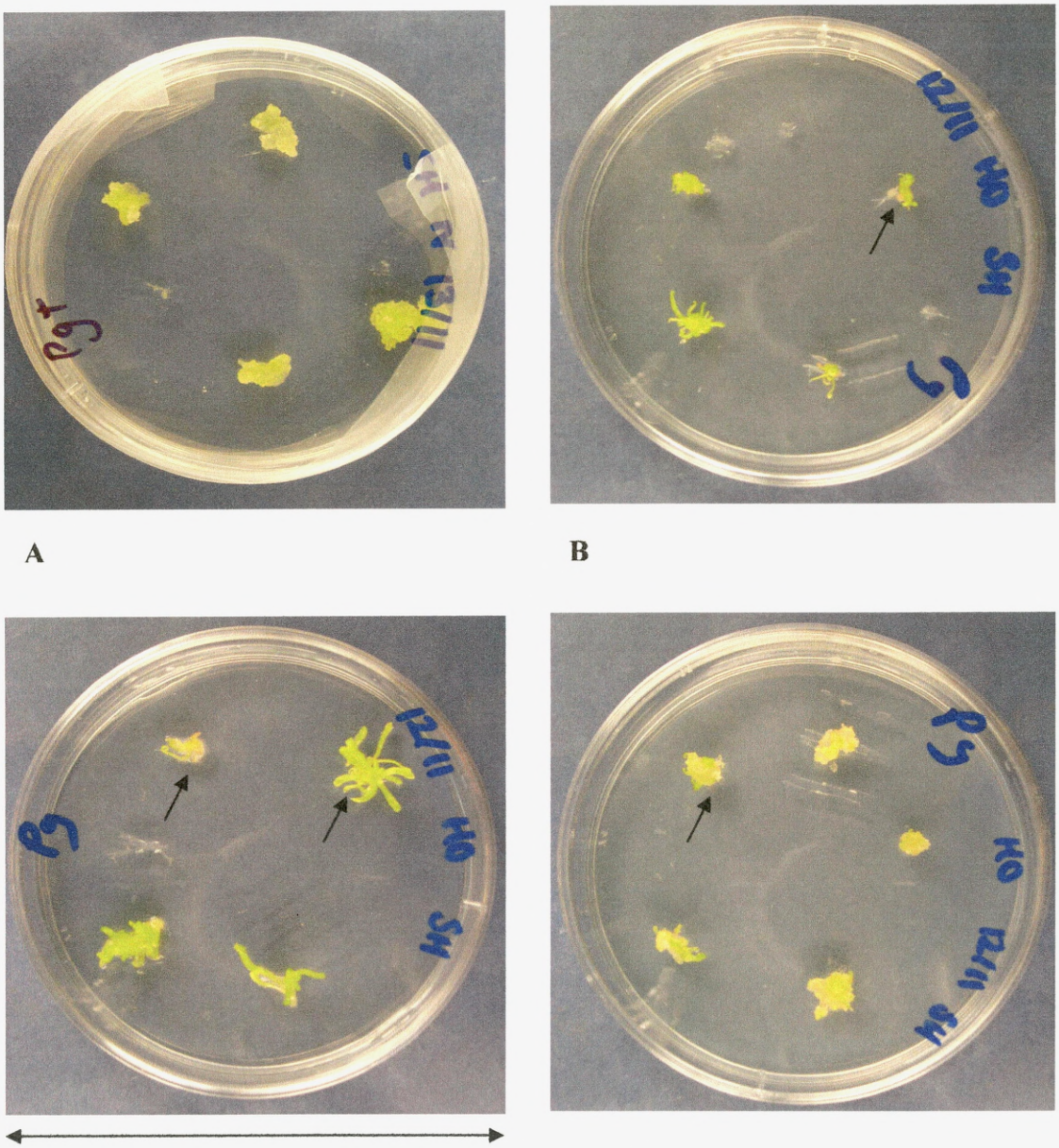


Figure 3.1: Growth analysis of *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. The data was measured every 3 days and the results are expressed as a fresh weight value. Errors are derived from the standard deviation of the data series.

The normal cell line (N) gained the greatest mass (see Table 3.1) in 30 days with the exponential growth phase beginning between days 10-14. Cell line N1 had a weight gain of 420%, cell line HO (380%) and cell line N3 (270%). Callus lines proliferated, in general, more rapidly than organogenic structures and as they contain more water, this enhanced their overall weight. The habituated organogenic cell line (HO) gained fresh weight (380%, note started with 50mg of material rather than 100mg) more rapidly than cell line N3, which was surprising as line N3 is callus. The organogenic habituated cell line had an exponential period of growth which started almost immediately, exponential growth in the fully habituated cell lines N1 and N3 started around day 5, however after day 14 there were extensive signs of cell necrosis, particularly in cell line N3.

3.3.2 General structural analysis



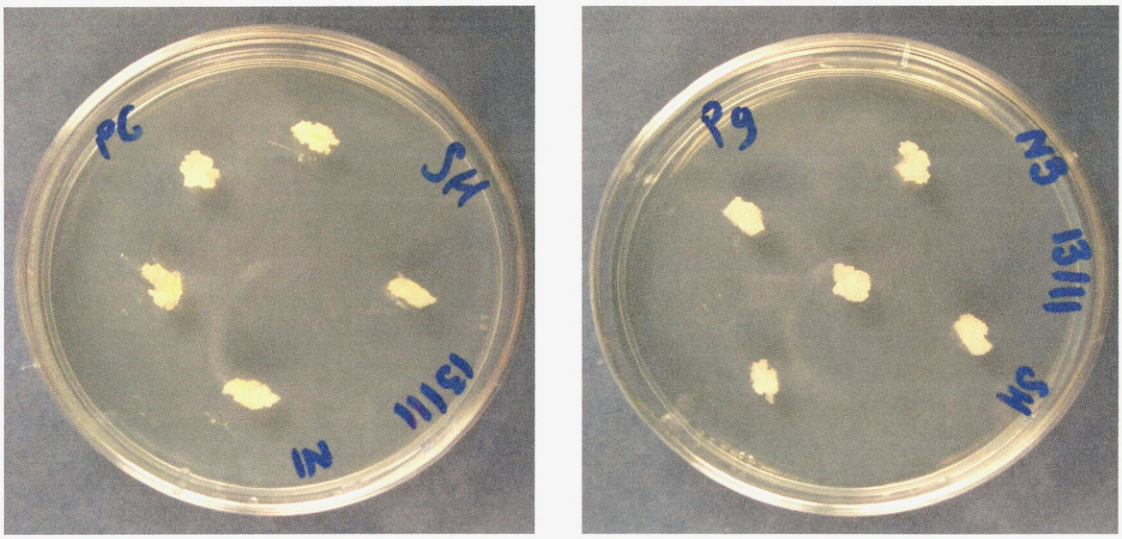
A

B

C

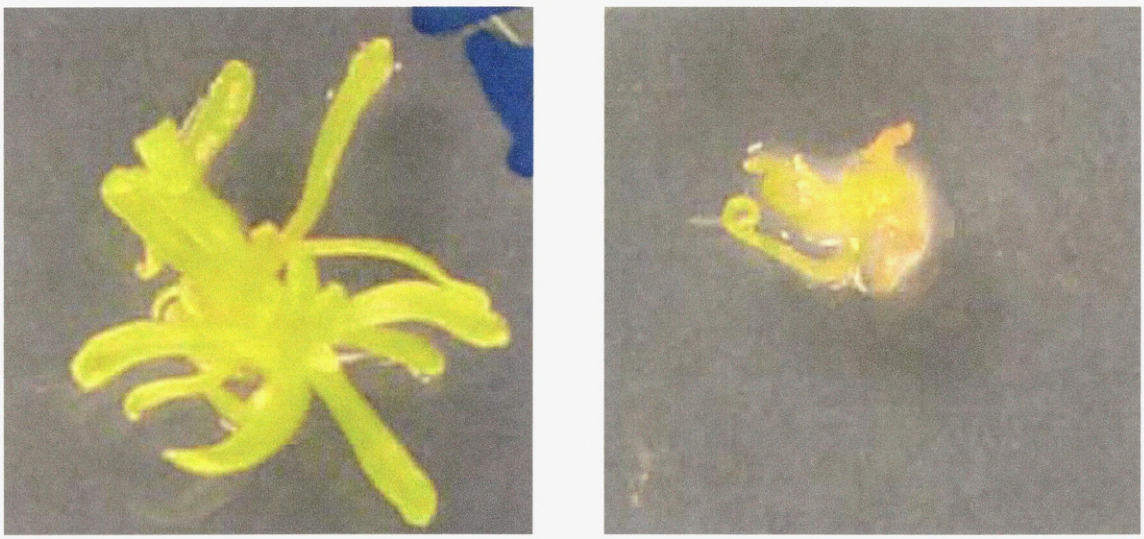
D

Scale: 9cm (Relative to Petri dish size)



E F

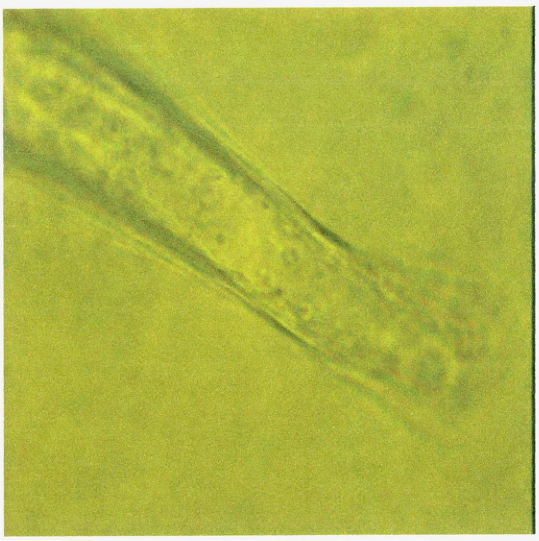
Figure 3.2: Digital images of *B. vulgaris* cell lines N, HO, N1 and N3. Image A: Normal non-habituated dedifferentiated cell lines; (N) maintained in medium supplemented with PGRs - callus appears green and compact. Images B-D: Organogenic (shoot producing) habituated cultures (HO) maintained on hormone free medium, highlighted in image (B), the cauliflower appearance of abnormal shoots, where shoots fail to elongate. Highlighted in (C), to the left, a shoot clump undergoing necrosis and, to the right, shoots that are abnormally elongated and have failed to broaden. Highlighted in (D) is an example of callus-like material formed at the base of the shoots. Image E: Fully habituated cell line (N1) completely dedifferentiated and proliferating as callus on hormone free medium; callus is white and very watery. Image F: Fully habituated cell line (N3), which like N1, is completely dedifferentiated material maintained on hormone free medium and callus is white and watery.



G H

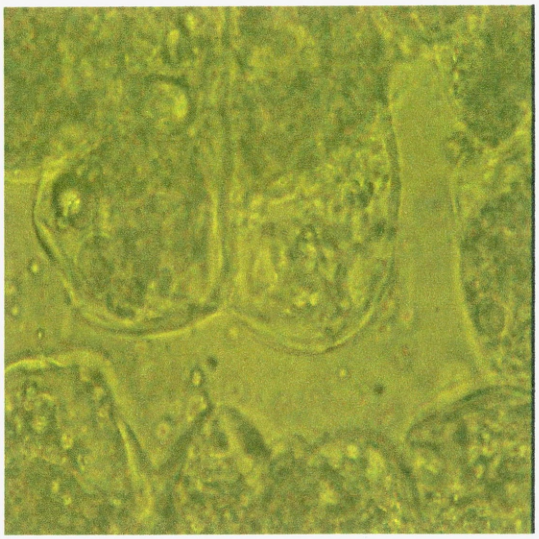
Figure 3.3: Close-up images (ca. 5X magnification digital) of abnormal shoot clusters in organogenic habituated cell line (HO). Image G shows the shoots are elongated, wrinkled and pale in colour and have curled tips and hyperhydricity evident. Image H is a closer view of the shoot necrosis, tips are browning and the clump has a very watery appearance.

3.3.3 *Light microscope analysis (X 100 magnification)*



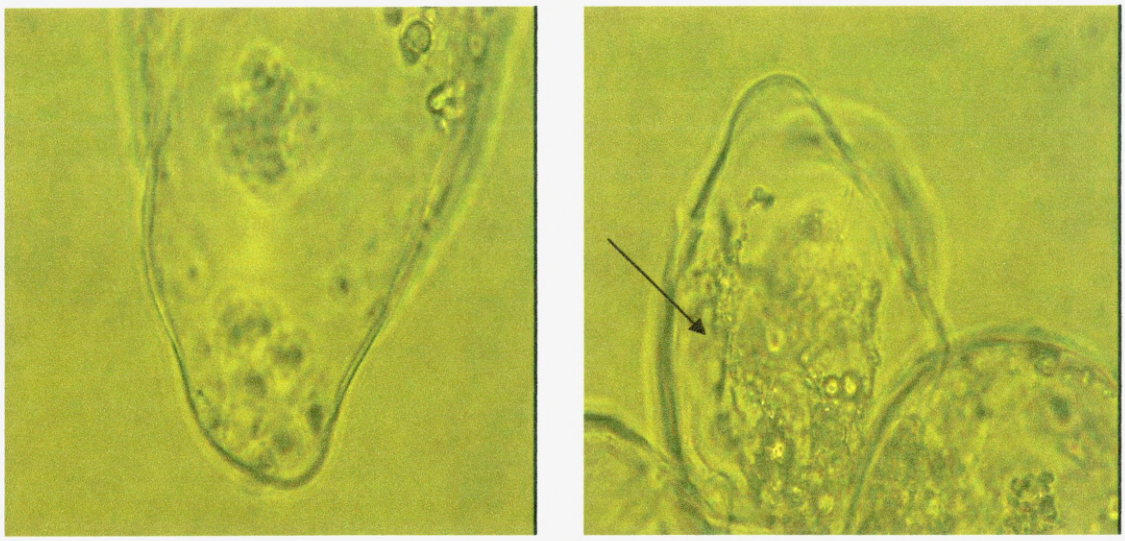
A

B



C

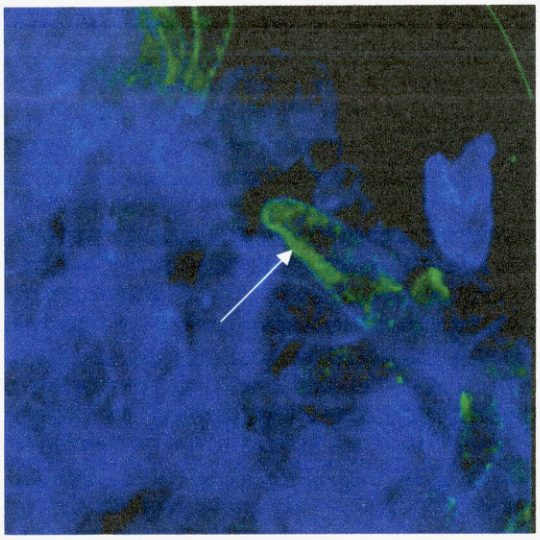
D



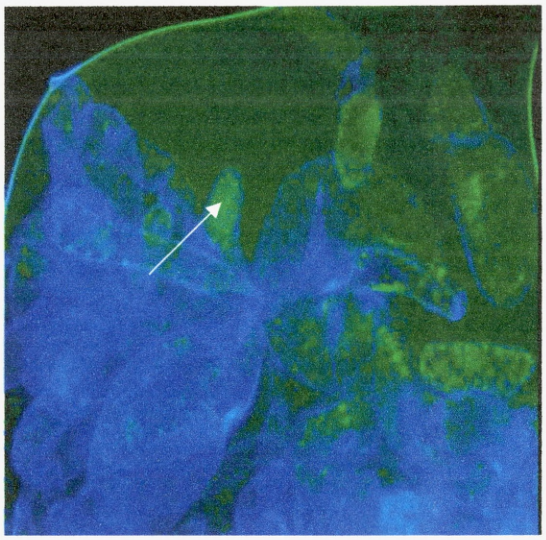
E F

Figure 3.4: Digital images collected from a light microscope (X 100). Images A and B: show that the normal non-habituated cells (N) are more elongated and more frequently elongated than the fully habituated cell lines. Image B: cells from the normal non-habituated line (N) showing that the membrane at the tip of the cell is possibly damaged and appears to be disturbed. Images C and D, of the fully habituated cells, show accumulated cellular debris but the cells are not elongated and are much smaller in comparison with the normal non-habituated cell lines. Image D, in particular, shows contents leaking from within the cell. Images E and F, taken from the fully habituated cell lines (N1+N3) show that the cells are irregularly shaped and accumulation of cellular debris is visible.

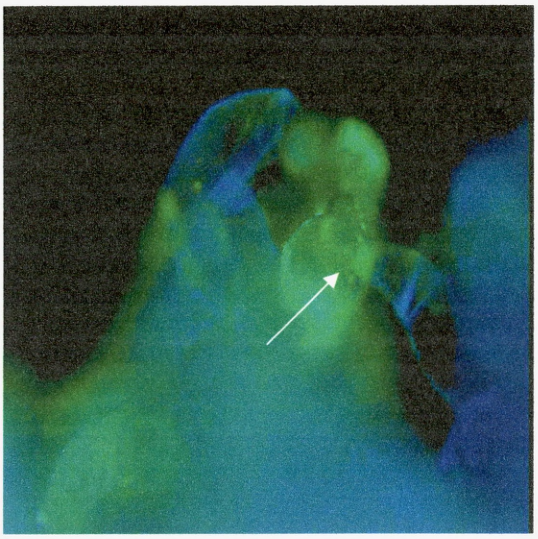
3.3.4 *Viability testing (X100 magnification) with Fluorescein Diacetate (FDA)*



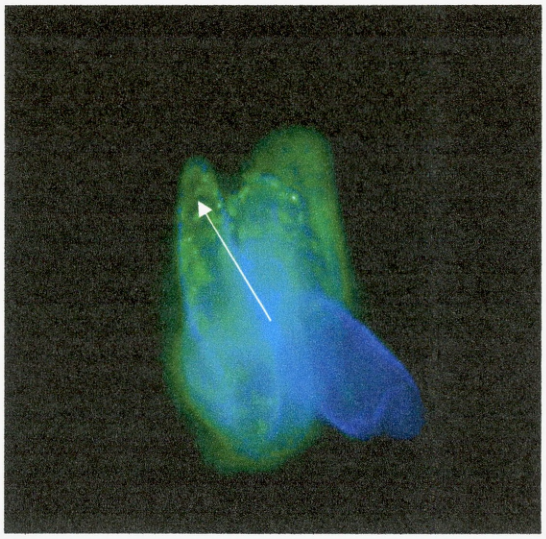
A



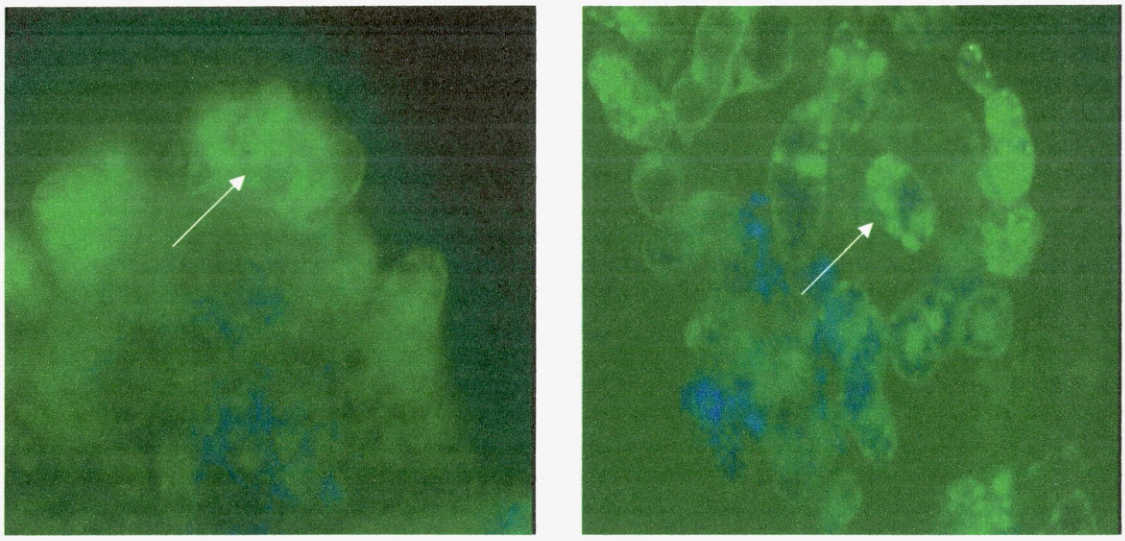
B



C



D



E **F**

Figure 3.5: Digital images of *B. vulgaris* captured under the microscope (X100 magnification) with a fluorescein diacetate (FDA) stain applied (blue colouration possibly attributed to highly necrotic atypical cells that are also non-viable, green colouration is indicative of viable cells, any orange coloured areas is due to chlorophyll interference). Image A and B: taken from the normal, non-habituated cell line (N) - viable cells are visibly elongated and high esterase activity (high fluorescent areas) visible at the edges of the membrane. Images C and D: taken from the organogenic cell line (HO) - cells again are elongated and there is very high esterase activity detected around the edges and tips of cells. Image E: taken from the fully habituated cell line (N1) - cells are smaller not elongated and esterase activity is denser than in the organogenic cell line (HO) and the normal cell line (N). Image F: taken from fully habituated cell line (N3) - cells are smaller and rounder in shape than the normal cell line with high esterase activity throughout the cell. Note stain application was used primarily to elucidate the morphological structures rather than viability.

***B. vulgaris* Cell line**	**Pigment**	**Organised structures**	**General characteristics**	**Hyperhydric behaviour**	**FDA viability**
Normal (N)	Light green Chlorophyll pigment	None	Cell elongation	None	Small areas appeared to fluoresce brightly indicating high esterase activity i.e. cell growth
Organogenic habituated (HO)	Light Green Chlorophyll pigment	Shoots only	Curled, wrinkled narrow shoot tips with cauliflower-like structures at the base. Completely dedifferentiated callus occasionally formed at the base of the shoot and cell elongation visible	Yes	Cell viability (esterase activity) was observed to be very high towards the tips of the cells, indicating that there are still active meristems
Fully habituated (N1)	No pigment	None	Watery, friable callus prone to extensive necrosis, small spherical cells	Yes	Small areas observed to fluoresce brightly indicating esterase activity (cell growth)
Fully habituated (N3)	No pigment	None	Watery, friable callus prone to extensive necrosis, small spherical cells	Yes	Small areas observed to fluoresce brightly indicating esterase activity (cell growth)

Table 3.2: Summary of morphological observations of habituated B. *vulgaris* cell lines.

3.4 BIOCHEMICAL ANALYSIS OF HABITUATED *B. VULGARIS*

The assays applied can be divided into the following three categories:

- Antioxidant assays
- Reactive oxygen species (ROS) detection
- Lipid peroxidation products

3.4.1 Antioxidant assays

Antioxidants are directly involved in the removal of reactive oxygen species and toxic products. They comprise enzymatic and non-enzymatic based protection systems. The following antioxidant enzymes were measured: catalase, peroxidase, superoxide dismutase, glutathione S-transferase and glutathione reductase and were always expressed on the basis of specific activity as the activity per milligram of protein. Non-enzymatic antioxidants comprised glutathione and ascorbate which were measured colorimetrically and their levels expressed as standard equivalents on the basis of fresh weight or protein as appropriate. Finally, the protein bound and non-protein bound sulphydryl group levels were measured.

3.4.1.1 Protein

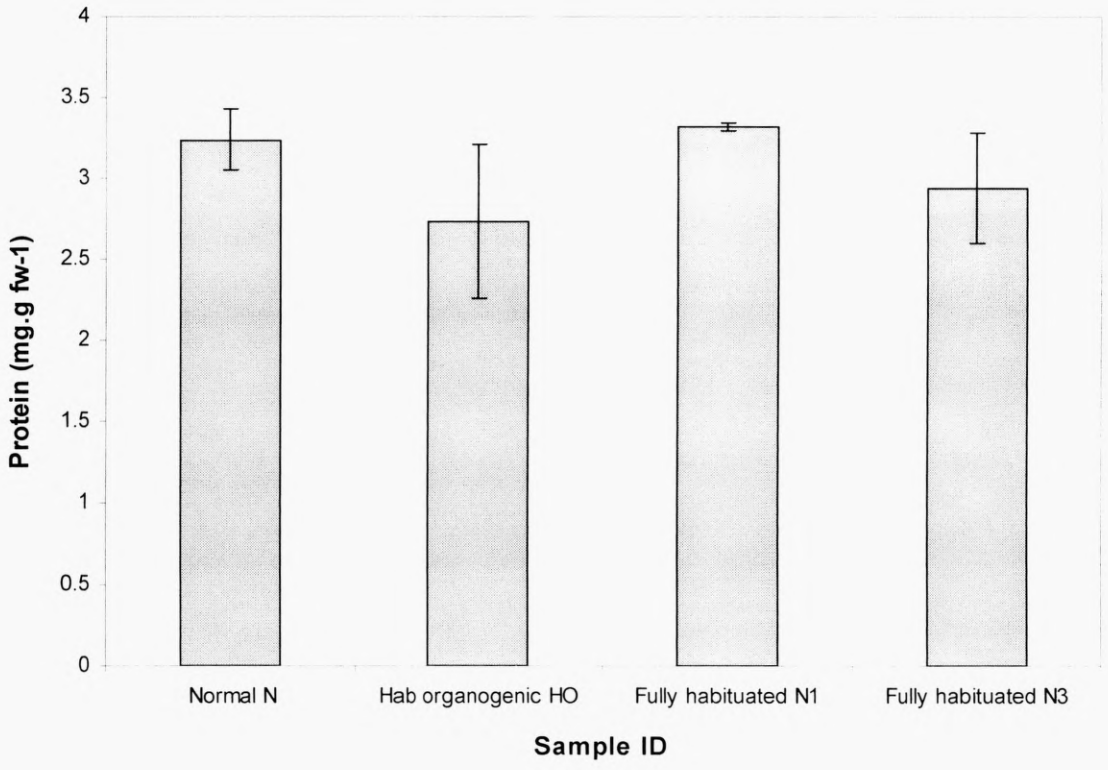


Figure 3.6: Protein content in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Protein levels are expressed as milligrams per gram of fresh weight material. Errors are derived from the standard deviations of the data series.

Protein levels between the four cell lines were not found to be significantly different ($P>0.05$, $F=2.76$). All ANOVA assumptions were satisfied for this data series.

3.4.1.2 Superoxide dismutase

No Cu,Zn-superoxide dismutase activity was detected in any of the four cell lines. Standard samples gave accurate and highly reproducible results, showing that the assay was viable.

3.4.1.3 Catalase

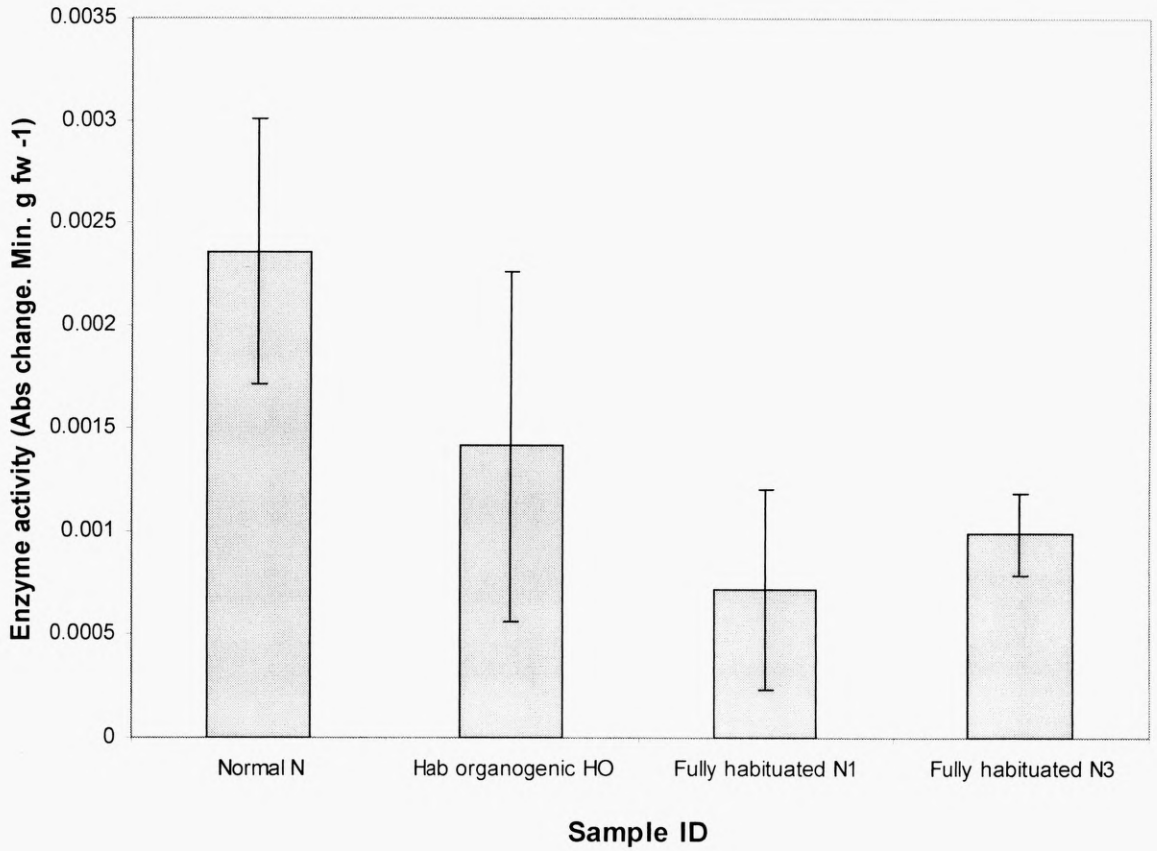


Figure 3.7: Catalase activity in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Enzyme activity was based on the change in absorbance per min per mg of protein. Errors are derived from the standard deviations of the data series.

Catalase activity was very low (0.00072-0.0026 Abs change.min.gfw $^{-1}$) in all four cell lines. Statistical analysis indicated that the data series did not have normal distribution and did not have equal variance, therefore ANOVA could not be used to analyse this data. Consequently, the less powerful Kruskall-Wallis test was used to analyse this non-parametric, highly variable data. The result was that no significant differences were observed between the four cell lines ($P>0.05$, $H=6.79$).

3.4.1.4 Peroxidase

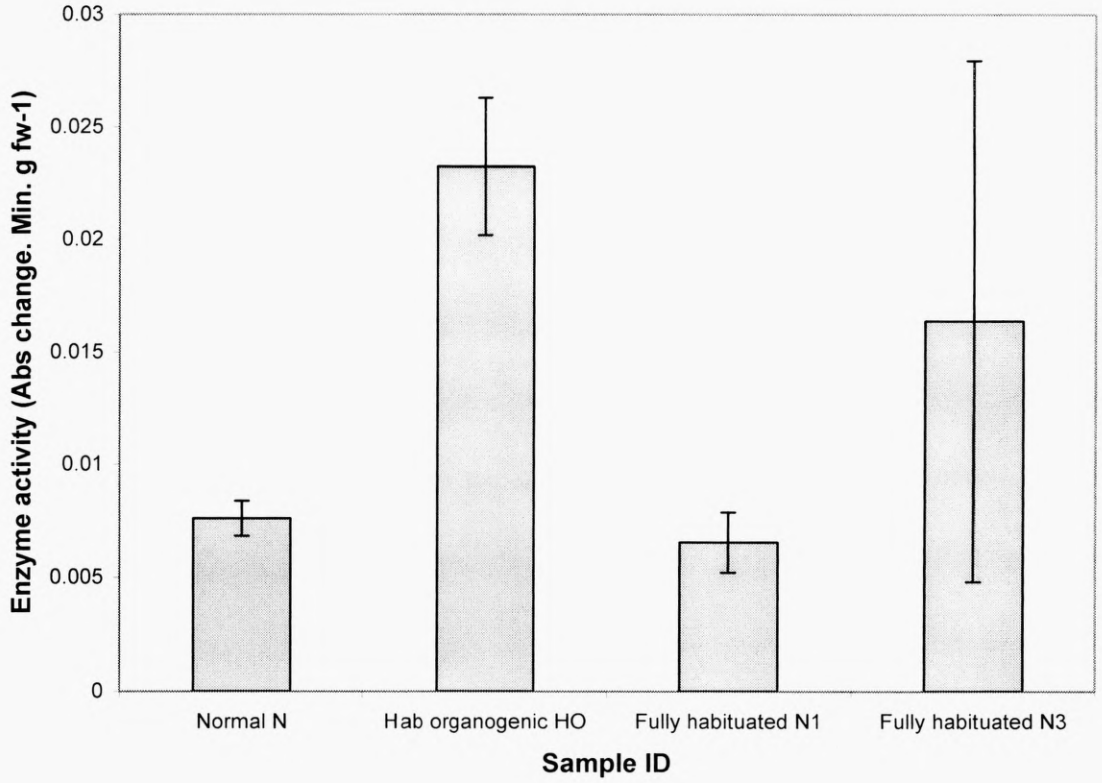


Figure 3.8: Peroxidase activity in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Enzyme activity was based on the change in absorbance per min per mg of protein. Errors are derived from the standard deviations of the data series

Peroxidase activity was detected in all four cell lines; however cell line N3 showed very variable results and one of the plates produced replicates with unusually high peroxidase activity, which resulted in the data series not having normal distribution or equal variance, therefore the Kruskal-Wallis test was applied for statistical analysis. The results showed that there was significantly higher peroxidase activity in the organogenic cell line (HO), ($P<0.05$, $H=14.10$).

3.4.1.5 Glutathione

Total glutathione

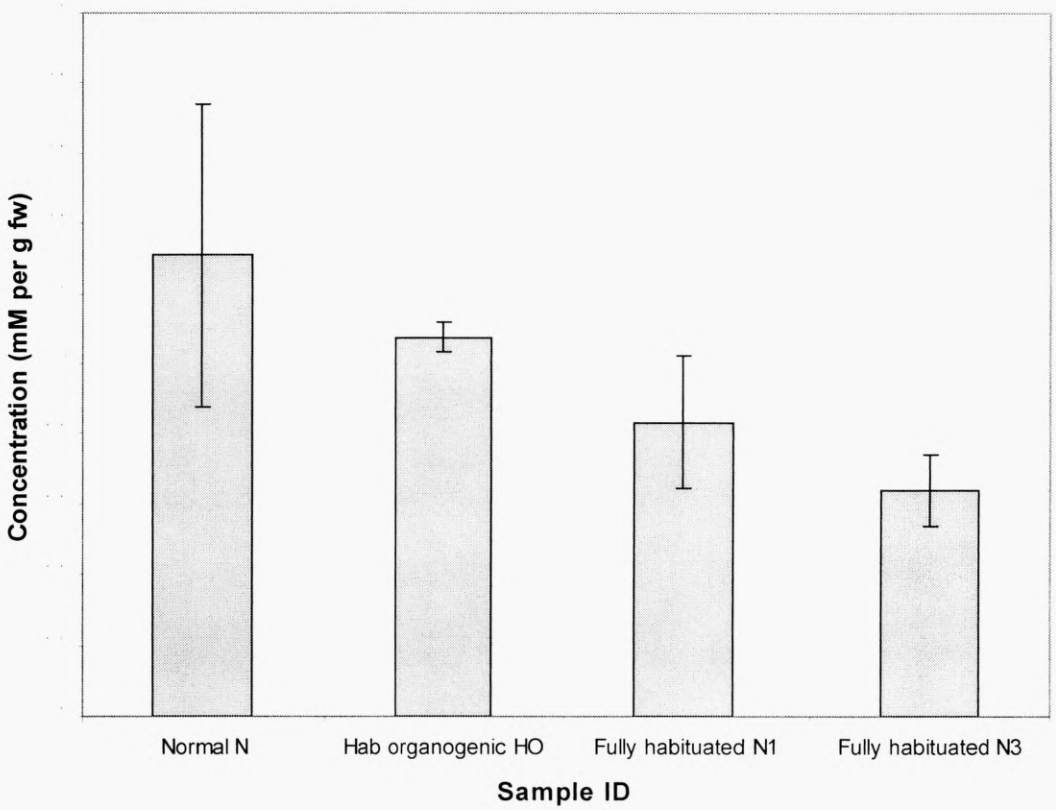


Figure 3.9: Total glutathione levels in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Concentration is based per g of fresh weight material. Errors are derived from the standard deviation of the data series.

The absolute values of glutathione showed that there were no significant differences between the four cell lines ($P<0.05$, $F=3.47$) and ANOVA assumptions were all satisfied for this data series.

Oxidised glutathione (GSSG)

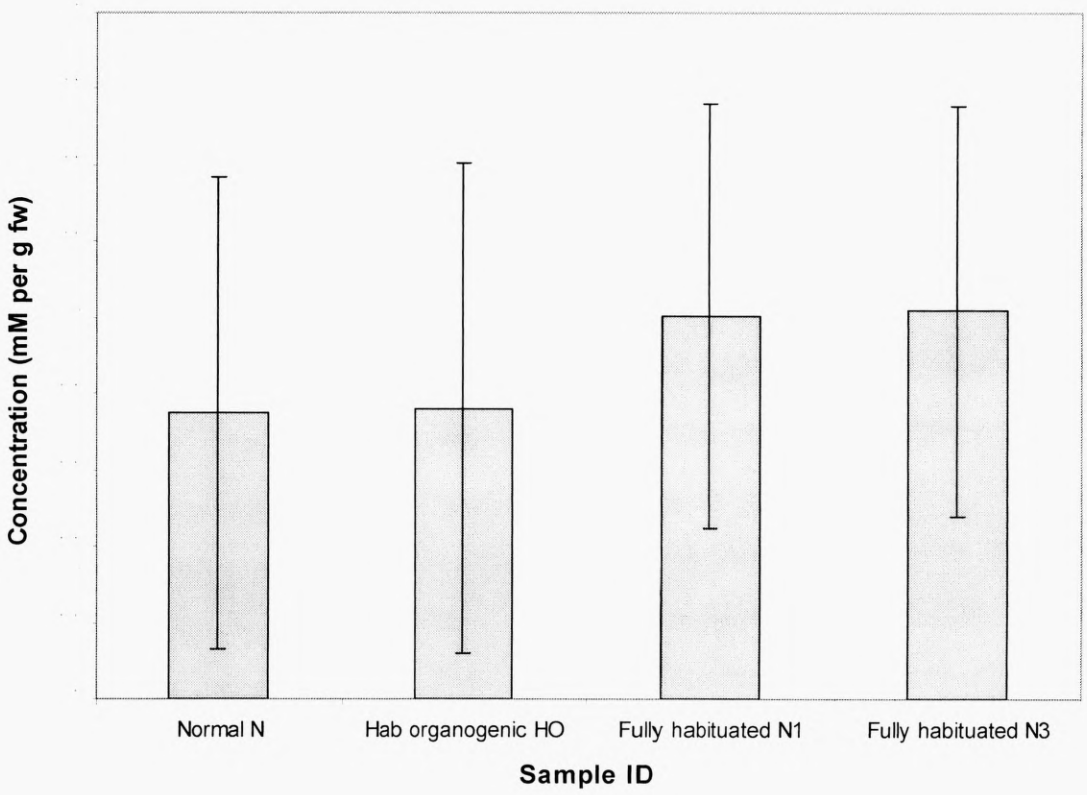


Figure 3.10: Oxidised glutathione (GSSG) levels in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Concentration is based per g of fresh weight material. Errors are derived from the standard deviations of the data series.

Oxidised glutathione (GSSG) levels did not significantly differ between the four cell lines ($P>0.05$, $F=2.03$) and all ANOVA assumptions were satisfied. Data collected was extremely variable, presumably due to the unstable nature of the products formed.

Comparison of reduced and oxidised glutathione

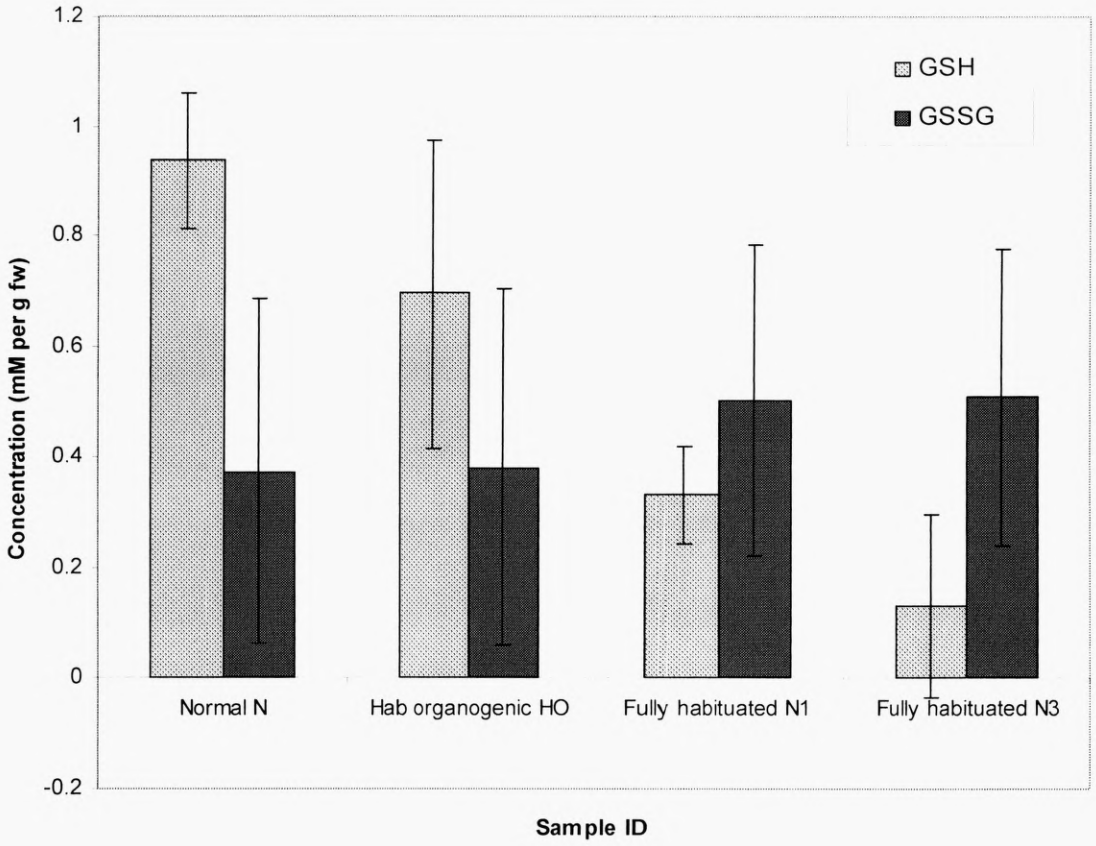


Figure 3.11: Comparison of reduced and oxidised glutathione in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Concentration is based per g of fresh weight material. Errors are derived from the standard deviations of the data series.

Sample ID	Glutathione redox status (ratio)
Normal (N)	0.7143
Habituated organogenic (HO)	0.646
Fully habituated (N1)	0.397
Fully habituated (N3)	0.204

Table 3.3: Glutathione redox status in *B. vulgaris* cell lines

The ratio between reduced and oxidised glutathione (Table 3.3) changed in the fully habituated cell lines N1 and N3 presumably due to the higher accumulation of

oxidised glutathione Also, the pool of available glutathione appears to decrease dramatically as the state of habituation progresses. The calculated redox status of GSH/GSSG (Table 3.3) decreases as the neoplastic stage progresses.

3.4.1.6 Ascorbate

Total ascorbate

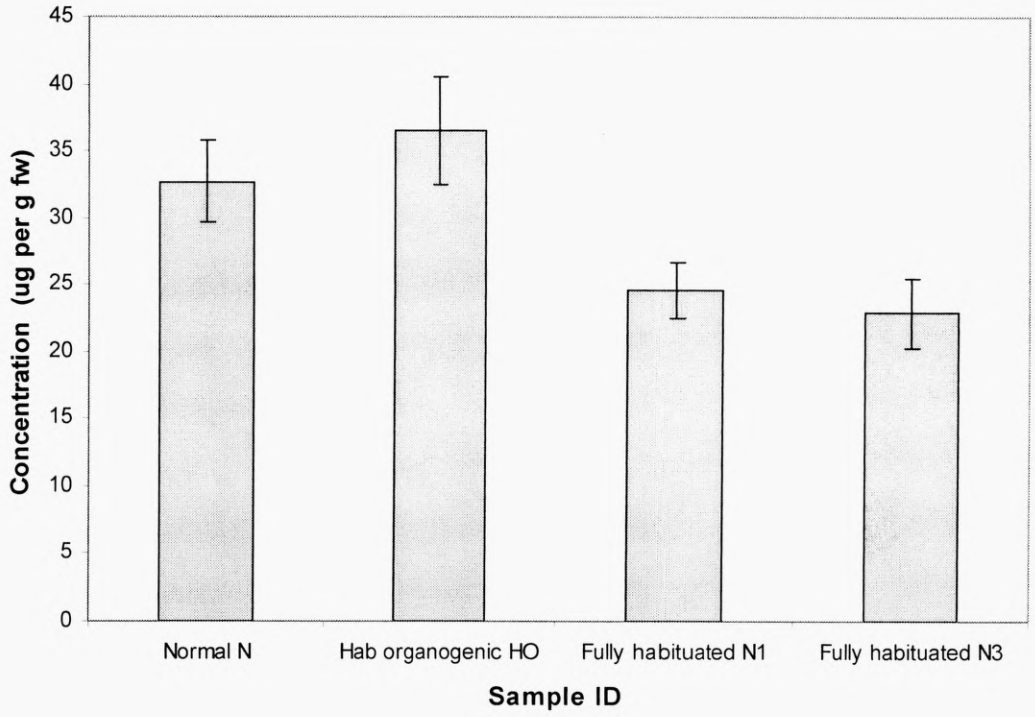


Figure 3.12: Total ascorbate and dehydroascorbate levels in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Concentration is based per g of fresh weight material. Errors are derived from the standard deviations of the data series.

There are significant differences between the cell lines as total ascorbate was significantly lower in the two non-organogenic habituated lines (N1 and N3), ($P<0.01$, $F=17.99$) and there were no differences between the other two cell lines. All ANOVA assumptions were satisfied for this data series.

Reduced ascorbate

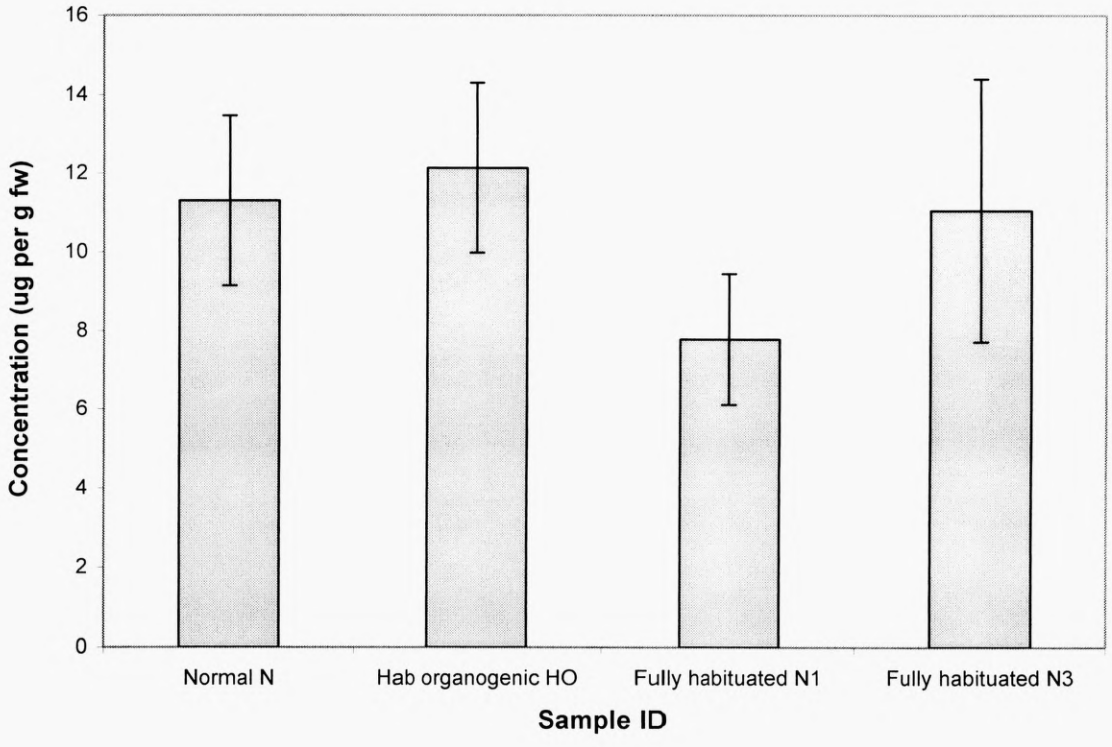


Figure 3.13: Ascorbate levels in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Concentration is based per g of fresh weight material. Errors are derived from the standards deviations of the data series.

There were significantly lower levels of ascorbate in one of the non-organogenic, fully habituated cell lines (N1) ($P<0.05$, $F=6.96$). There were no significant differences between the other three cell lines and all ANOVA assumptions were satisfied.

Comparison of reduced and oxidised ascorbate

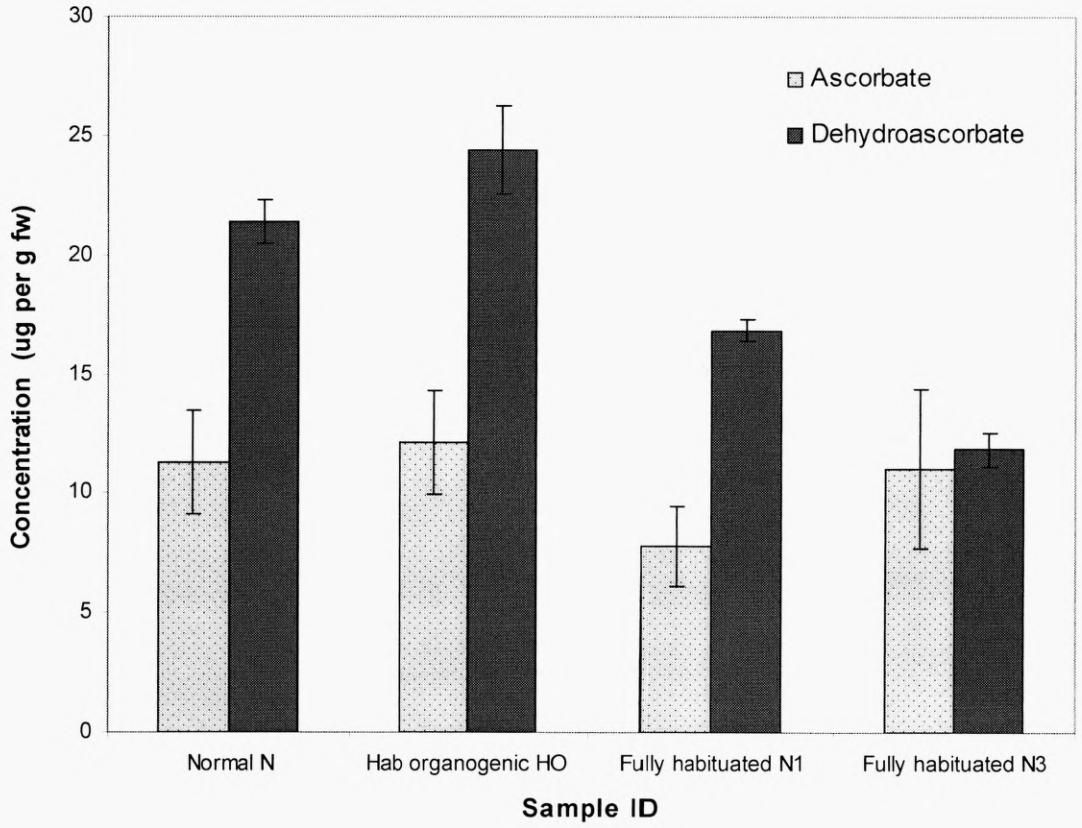


Figure 3.14: Comparison of ascorbate and dehydroascorbate in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Concentration is based per g of fresh weight material. Errors are derived from the standard deviations of the data series

Sample ID	Ascorbate redox status
Normal (N)	0.3456
Habituated organogenic (HO)	0.3322
Fully habituated (N1)	0.3160
Fully habituated (N3)	0.4873

Table 3.4: Ascorbate redox status of *B. vulgaris* cell lines

The oxidation, reduction ratio of ascorbate (see Table 3.4), like the redox state of glutathione, has a large effect on the redox potential of other cellular components. The ascorbate and the glutathione cycle cooperate in conjunction with each other and as such, changes in glutathione availability can have implications on the ascorbate cycle. The redox status of three of the cell lines (N, HO and N1) was very similar but N3, one of the fully habituated cell lines, had a higher redox state (0.48) and had lower levels of oxidised ascorbate.

3.4.1.7 Sulphydryl groups

Total sulphydryl groups

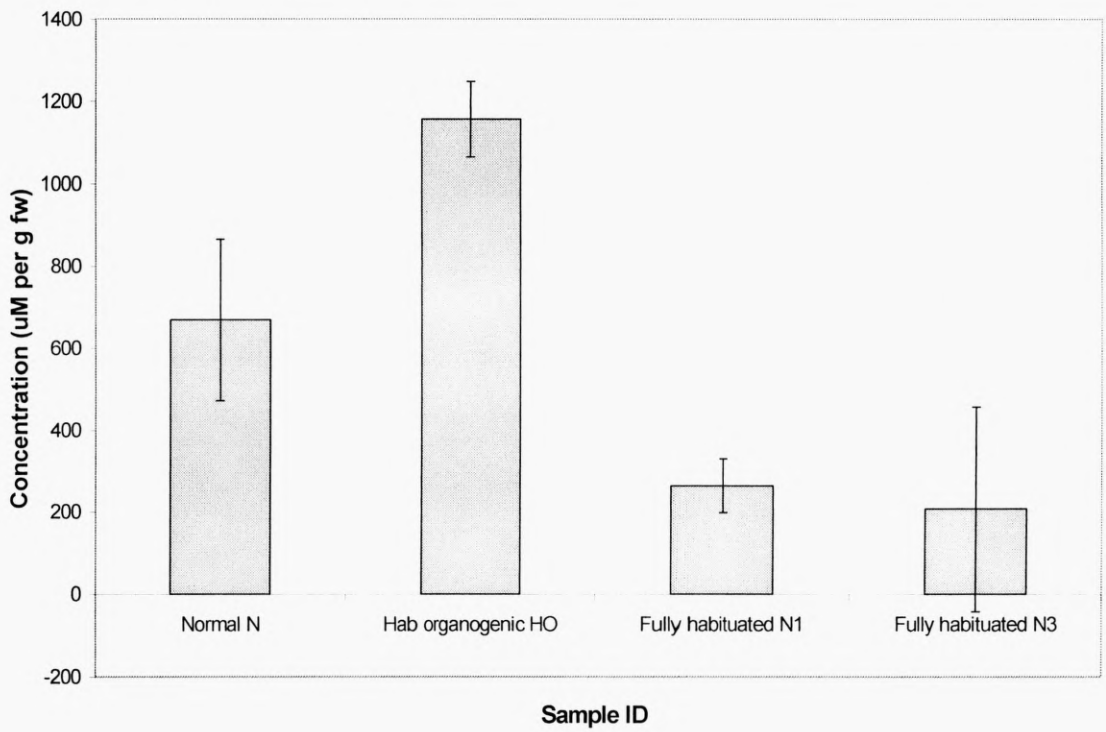


Figure 3.15: Protein-bound sulphydryl groups in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Concentration is based per g of fresh weight material. Errors are derived from the standard deviations of the data series.

The data series did not have equal variances; therefore the Kruskall-Wallis method was used for statistical analysis. The results showed that there were significant differences between the cell lines ($P<0.01$, $H=29.57$). Cell line HO was significantly different from lines N, N1 and N3 though N1 and N3 were not significantly different from each other.

Non-protein bound sulphydryl groups

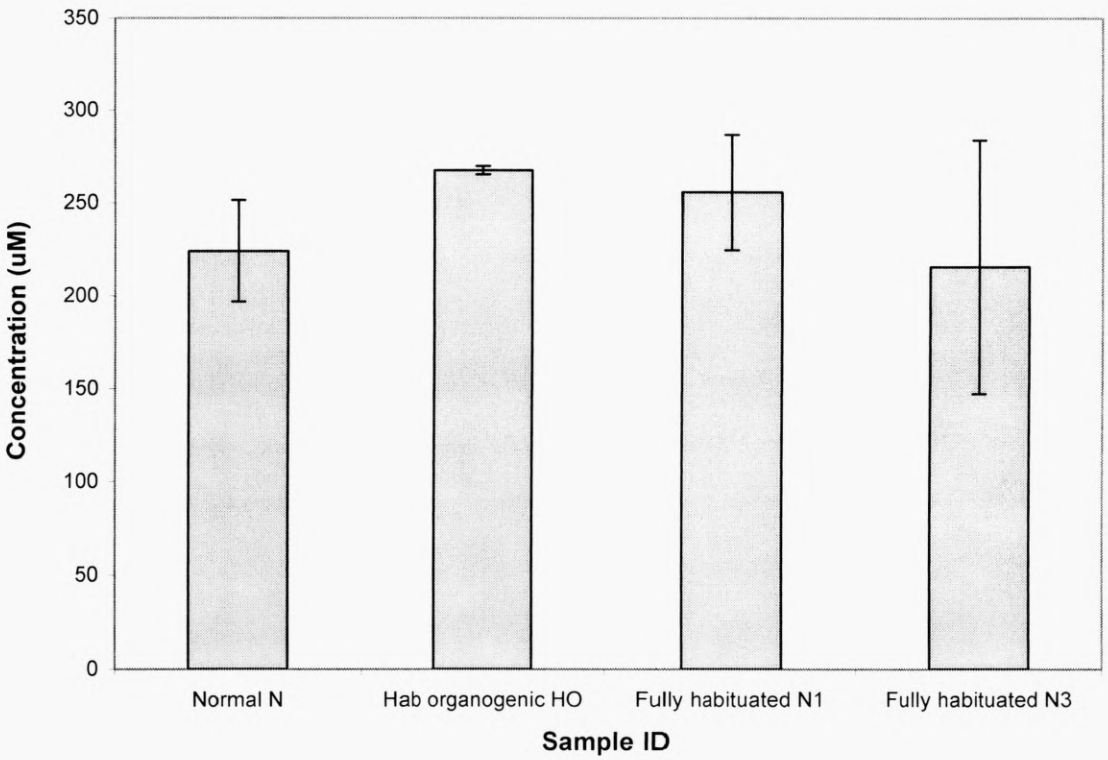


Figure 3.16: Non protein-bound sulphydryl groups in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Concentration is based per g of fresh weight material. Errors are derived from the standard deviations of the data series.

For the non-protein sulphydryl groups there were no significant differences between the four *B. vulgaris* cell lines ($P>0.05$, $F=2.88$) and all ANOVA assumptions were satisfied. This data shows a similar pattern to that of the glutathione (GSH) assay,

which could be expected, as glutathione is included in this group of non-protein bound sulphydryl.

Comparison of protein and non-protein bound sulphydryl groups

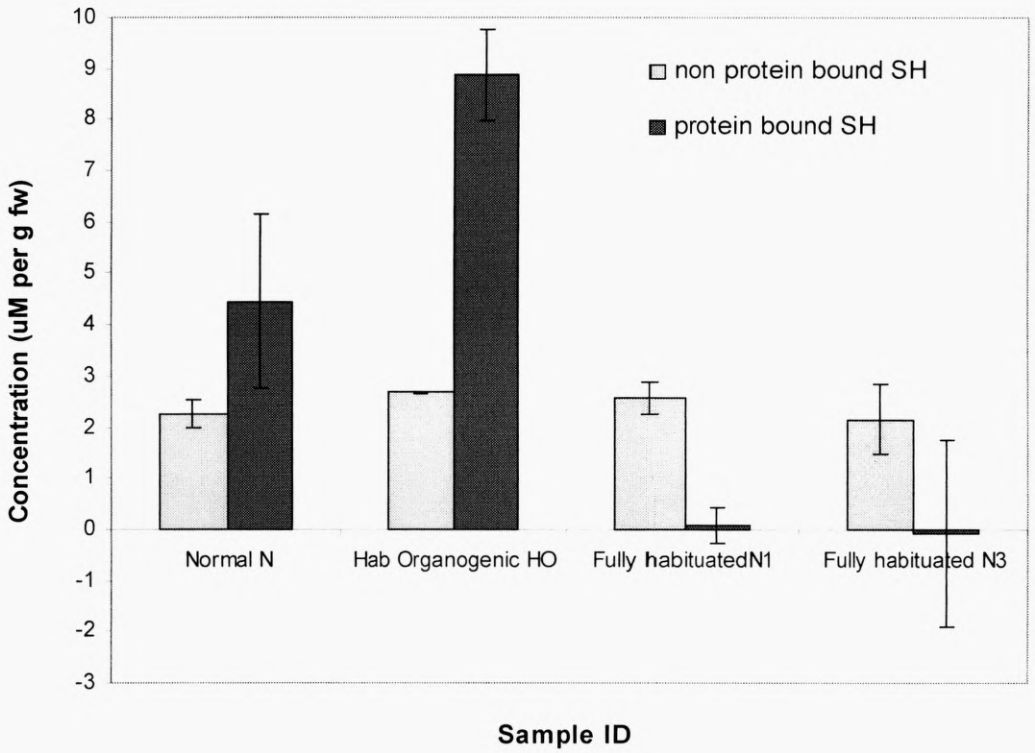


Figure 3.17: Protein-bound sulphydryl groups in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Concentration is based per g of fresh weight material. Errors are derived from the standard deviations of the data series.

Reduced glutathione (GSH) is one of the main types of non-protein bound sulphydryl groups and the data is similar to that derived from the reduced glutathione assay (see section 3.4.1.5). However, protein-bound SH groups in the fully habituated cell lines N1 and N3 are significantly lower, in fact almost completely depleted. This would indicate that there may be significant damage, potentially to SH- containing proteins, for which GSH can act as a preferential substrate for ROS in these cell lines.

3.4.1.8 Glutathione reductase

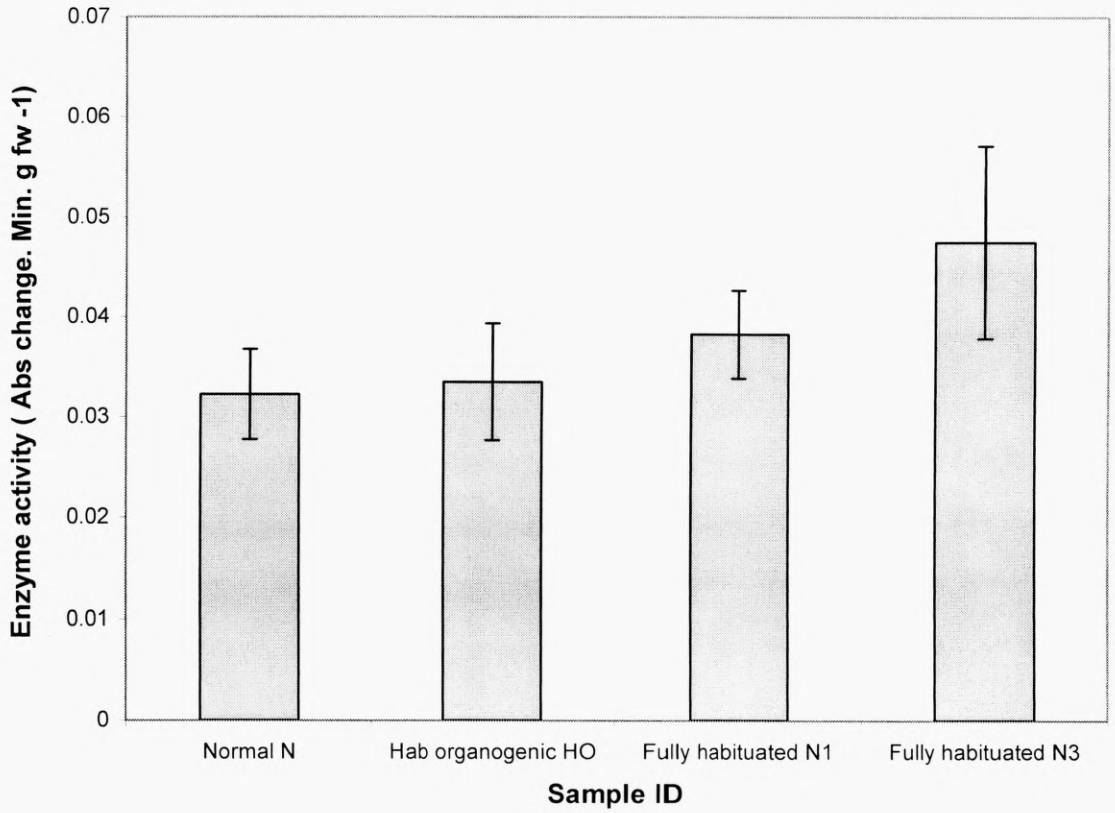


Figure 3.18: Glutathione reductase activity in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Enzyme activity was based on the change in absorbance per min per mg of protein. Errors are derived from the standard deviations of the data series

The data series did not have normal distribution, therefore the Kruskall-Wallis test was applied, but there were no significant differences in glutathione reductase activity between the four cell lines ($p>0.05$, $H=2.11$).

3.4.1.9 Glutathione S-transferase

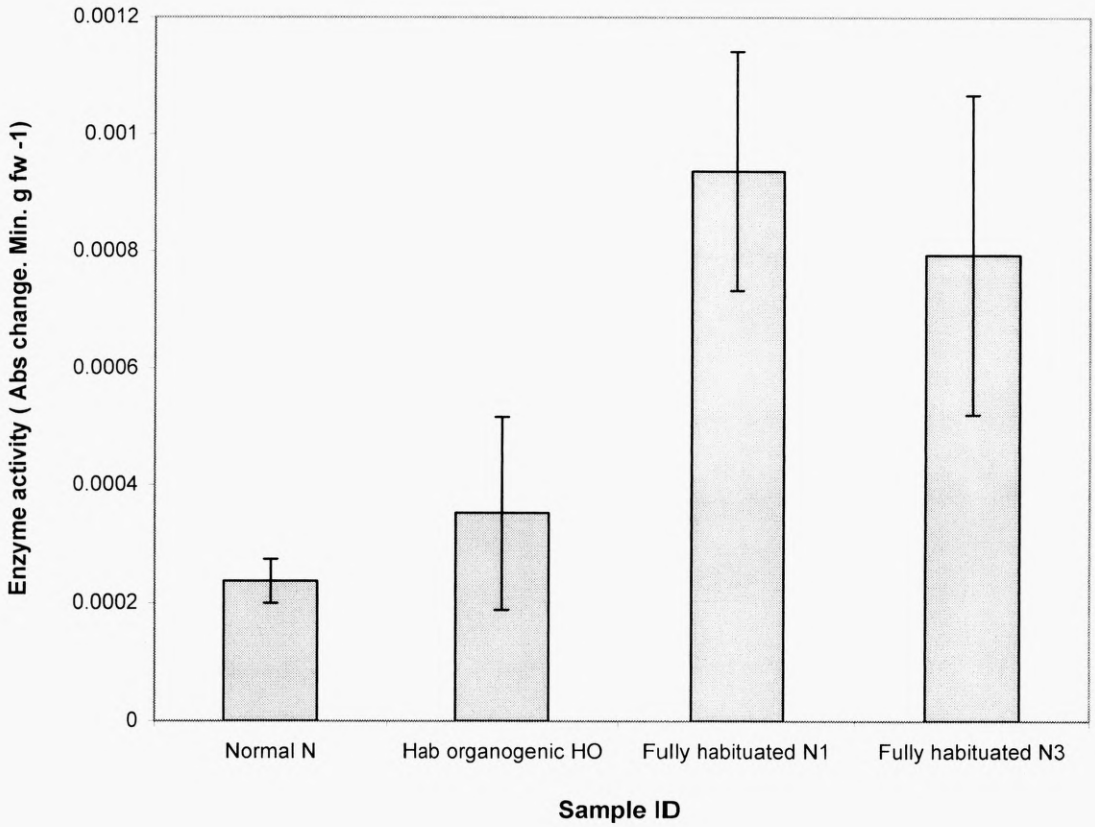


Figure 3.19: Glutathione S-transferase activity in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Enzyme activity was based on the change in absorbance per min per mg of protein. Errors are derived from the standard deviations of the data series.

Glutathione S-transferase activity was significantly higher in the two fully habituated cell lines (N1 and N3), ($P<0.01$, $F=25.19$). The normal cell line (N) and the organogenic habituated line (HO) did not significantly differ from each other and ANOVA assumptions were satisfied for this data series.

3.4.2 Reactive oxygen species (ROS)

3.4.2.1 Hydrogen peroxide

The assay was capable of detecting hydrogen peroxide within the range of 10-200µL/mL (3% H_2O_2), however in all four *B. vulgaris* cell lines no hydrogen peroxide was detected.

3.4.2.2 Hydroxyl radical

Hydroxyl radical activity was detected using DMSO as a radical trap to produce methane, which is measured by gas chromatography. No hydroxyl radical activity was found in any of the *B. vulgaris* cell lines. An interesting observation was that cell line N, the normal callus line, produced methane. When the values were compared to the control samples it appeared the control that contained cell line N medium and DMSO only, also seemed to produce methane. The values from the controls and the samples did not significantly differ ($P>0.05$, $F=2.27$), therefore it was concluded that the methane produced from cell line N samples was not as a result of hydroxyl activity generated from the sample. This observation suggests that a component of the medium itself that was reacting with DMSO to produce methane rather than hydroxyl radicals from the sample. This is indeed a possibility in complex media systems that contain transition metal cations (Benson, 2000).

3.4.3 Lipid peroxidation products

Lipid peroxidation is a complex process wherein unsaturated lipids, situated in the lipid membrane are attacked by ROS. The reaction starts off with hydrogen abstraction and after several complex steps results in the formation of conjugated dienes and lipid hydroperoxides. The latter degrade further to produce a variety of products, including alkanals, alkenals, hydroxyalkenals, ketones and alkanes.

3.4.3.1 Conjugated dienes

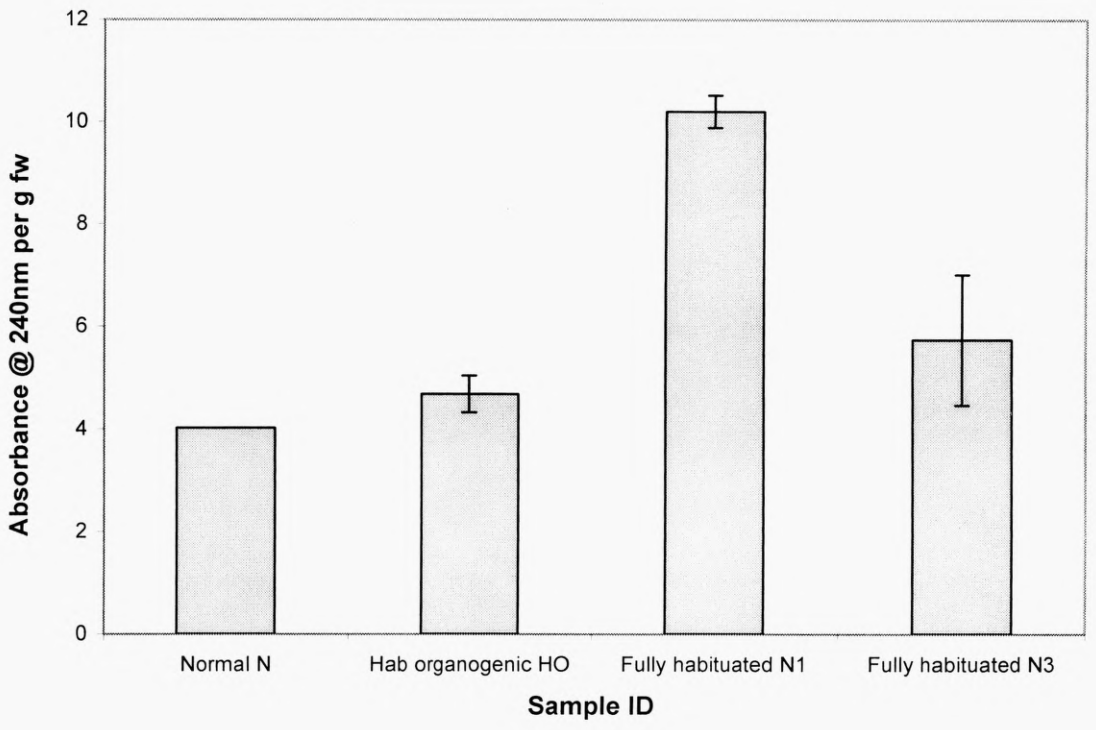


Figure 3.20: Conjugated dienes in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Concentration is based on an absorbance measured at 240nm per g of fresh weight material. Errors are derived from the standard deviations of the data series.

There were significant differences between the four cell lines and the non-organogenic habituated line N1 had significantly higher levels of conjugated dienes

compared with the other three cell lines ($P<0.01$, $F=7.06$). The data for cell line N3 although slightly higher was not significantly different. ANOVA assumptions were all satisfied.

3.4.3.2 Lipid hydroperoxides

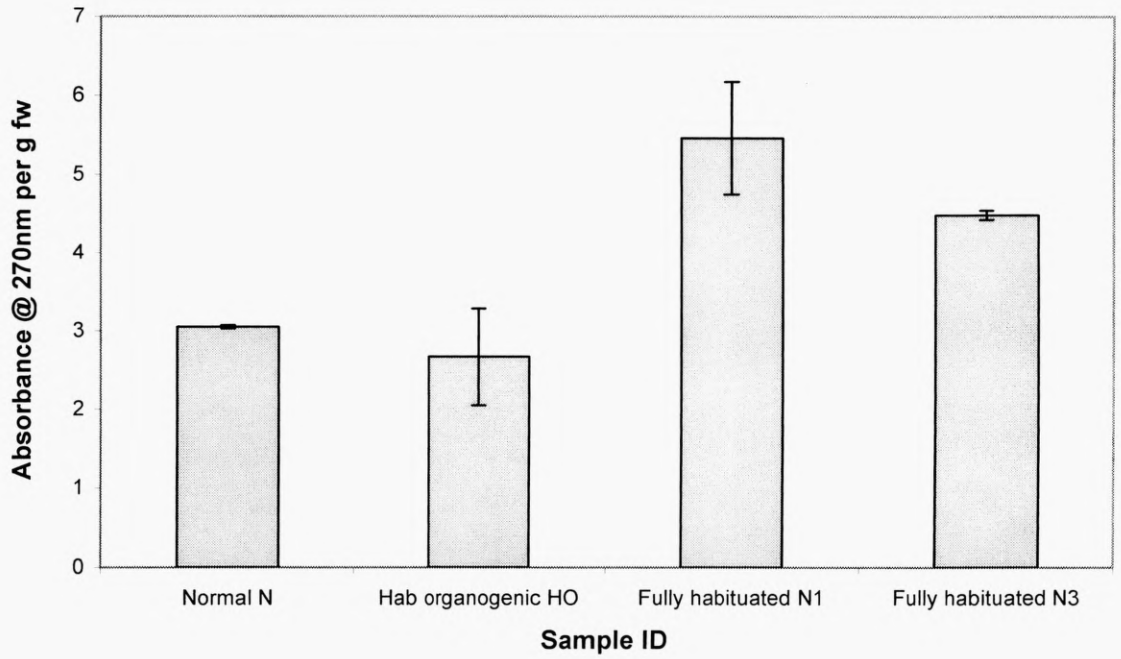


Figure 3.21: Lipid hydroperoxides in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Concentration is based on an absorbance measured at 270nm per g of fresh weight material. Errors are derived from the standard deviations of the data series.

The data indicated that there were significant differences between the cell lines ($P<0.05$, $F=3.99$) for lipid hydroperoxides. The habituated cell lines N1 and N3 were significantly different from the organogenic habituated cell line HO and the normal cell line N. All ANOVA assumptions were satisfied for this data series.

3.4.3.3 Malondialdehyde and 4-hydroxyalkenals assay (LPO-586 assay)

Total MDA + 4-hydroxyalkenals

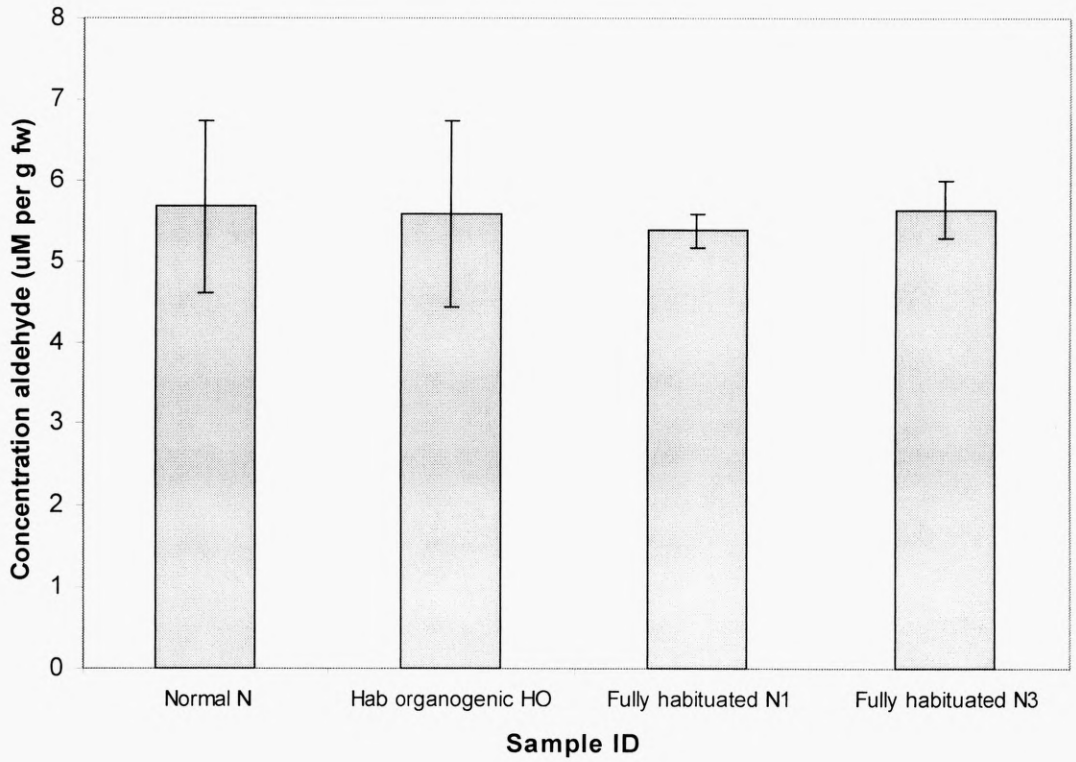


Figure 3.22: Total MDA + 4-hydroxyalkenals in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Concentration is based on an absorbance detected at 586nm and converted to μM concentration from a standard curve; the concentration is expressed per g of fresh weight material. Errors are derived from the standard deviations of the data series

There were no significant differences between any of the cell lines in the concentration of total MDA and 4-hydroxyalkenals ($P>0.05$, $F= 0.18$) and all ANOVA assumptions were satisfied for this data series.

MDA

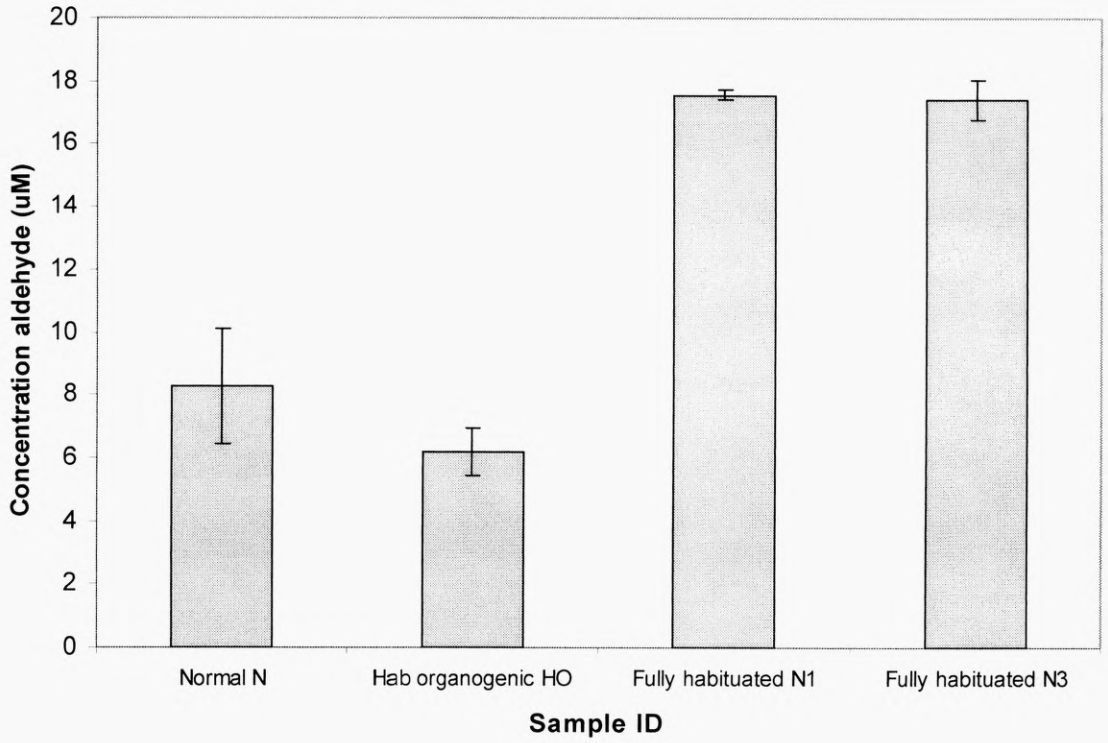


Figure 3.23: MDA in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Concentration is based on an absorbance detected at 586nm and converted to μM concentration from a standard curve; the concentration is expressed per g of fresh weight material. Errors are derived from the standard deviations of the data series

The data showed that there were significant differences in the total concentration of MDA in the four cell lines ($P<0.01$, $F=36.45$). The fully habituated cell lines N1 and N3 had the highest concentration and there were no significant differences between the normal cell line (N) and the organogenic cell line (HO). All ANOVA assumptions were satisfied for this data series.

Comparison of malondialdehyde and 4-hydroxyalkenals

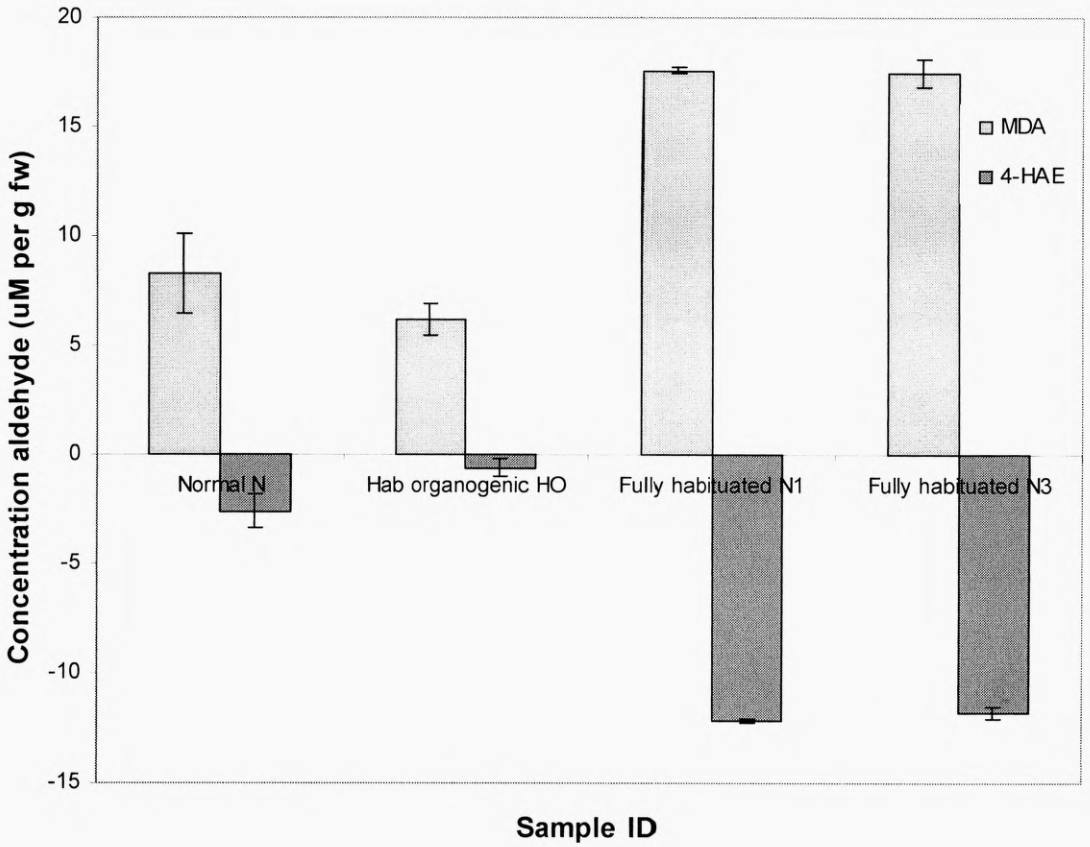


Figure 3.24: Comparison of MDA and 4-hydroxyalkenals in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Concentration is based on an absorbance detected at 586nm and converted to μM concentration from a standard curve; the concentration is expressed per g of fresh weight material. Errors are derived from the standard deviations of the data series

This lipid peroxidation product assay (LPO-586) is designed to determine the levels of malondialdehyde and 4-hydroxyalkenals present in a sample. The level of each type of aldehyde is calculated by determining, firstly, the level of total aldehydes (MDA + 4-hydroxyalkenals) and then secondly determining MDA alone. The "MDA only" values were subtracted from the "total aldehydes" values to give a figure for the 4-hydroxyalkenals. During this assay, the values ascertained from the total aldehydes assay were lower than the values for the MDA only values, which presented huge problems in the interpretation, as this produced negative values for 4-

hydroxyalkenals. The results can possibly be explained by one of two theories: either the figure for "total" aldehydes is very low or the amount of "MDA only" has been greatly overestimated. There is also evidence to suggest an interference in both assays, as there was an unidentified orange chromophore formed in all samples (see Appendix 6.2). This observation will be discussed in more detail later (see section 3.7.2.4). Conclusions could not be drawn from this assay due to the negative values; therefore as a consequence, this assay was considered an invalid method for quantifying MDA and 4-HNEs in *B. vulgaris*.

3.4.3.4 TBARS assay

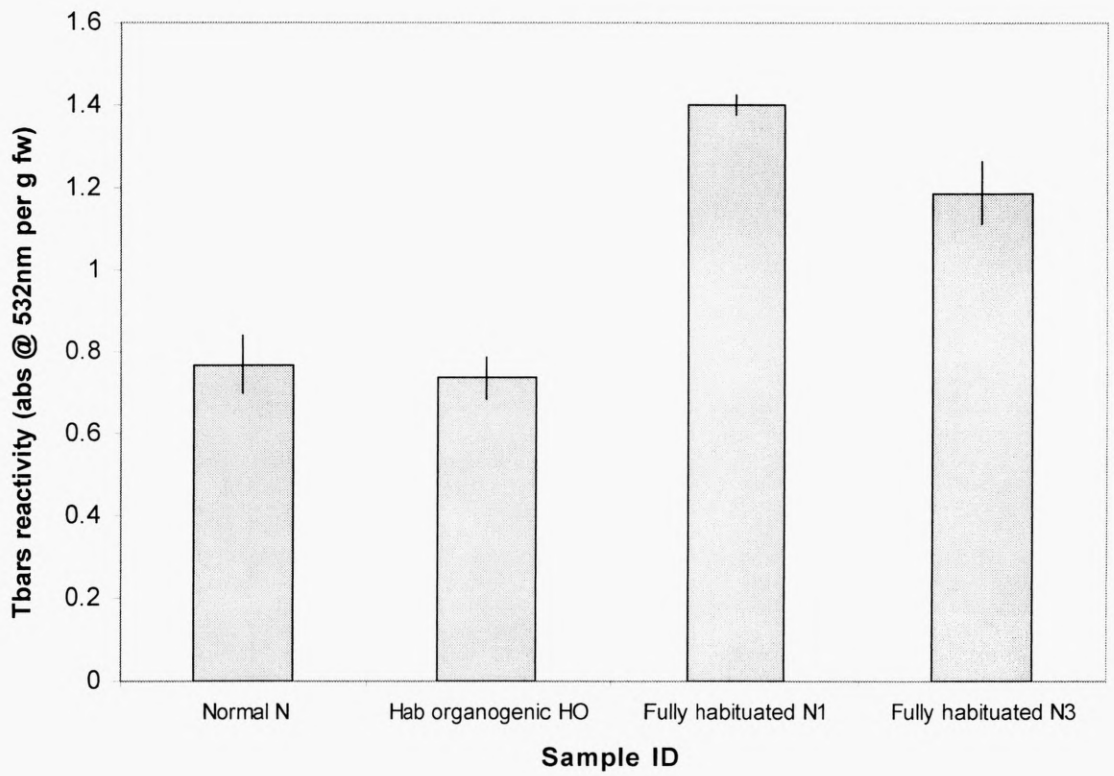


Figure 3.25: Thiobarbituric acid reactive species (TBARS) in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Concentration is based on an absorbance detected at 532nm and converted to μM concentration using the Beer-Lambert law; the concentration is expressed per g of fresh weight material. Errors are derived from the standard deviations of the data series.

There were significant differences between the cell lines ($P<0.01$, $F= 63.91$). The values for thiobarbituric reactive substances were higher in the two non-organogenic habituated cell lines (N1 and N3), but there were no significant differences between lines N and HO. All ANOVA assumptions were satisfied.

3.4.3.5 Schiff's bases

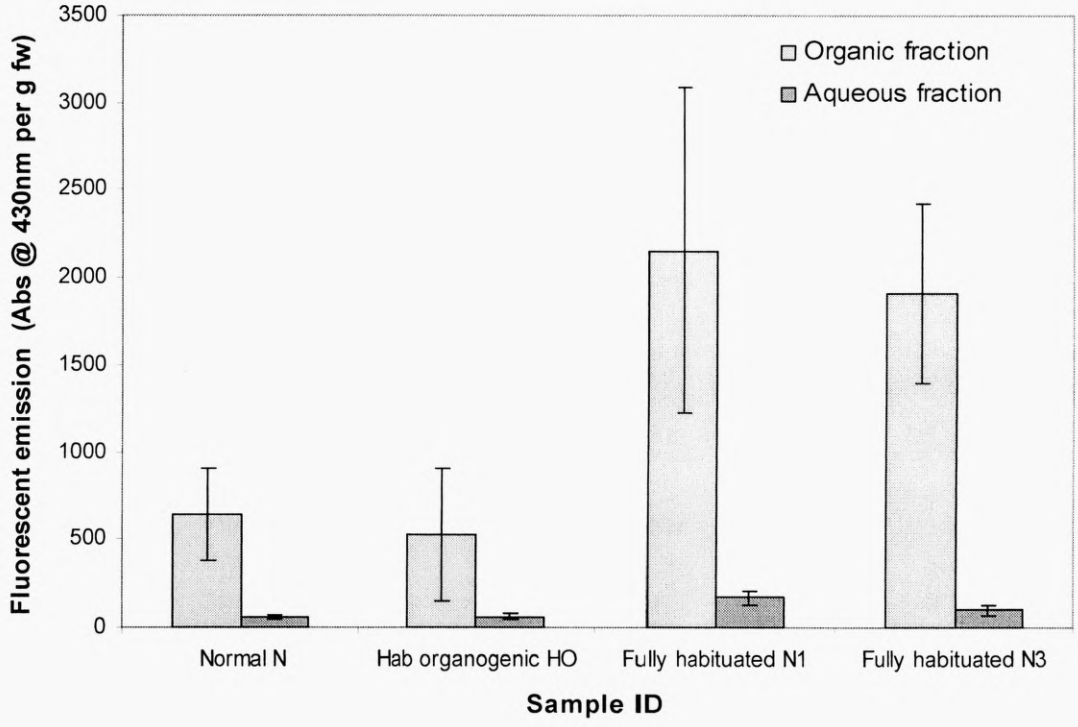


Figure 3.26: Schiff's bases in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Concentration is based on the fluorescent emission detected at 430nm and is expressed per g of fresh weight material. Errors are derived from the standard deviations of the data series.

The levels of Schiff's bases were higher in both organic and aqueous phases in the fully habituated cell lines (N1 and N3). Both sets of data did not have normal distribution or equal variances, therefore it was not possible to use ANOVA to statistically analyse the data. The Kruskall-Wallis test was used to analyse the non-

parametric data and the results showed that the differences between the cell lines were significant ($P<0.01$, $H=25.89$; $P<0.01$, $H=19.53$ for organic and aqueous phases respectively).

3.5 DNA CONTENT AND DNA METHYLATION STATUS

3.5.1 DNA and RNA content

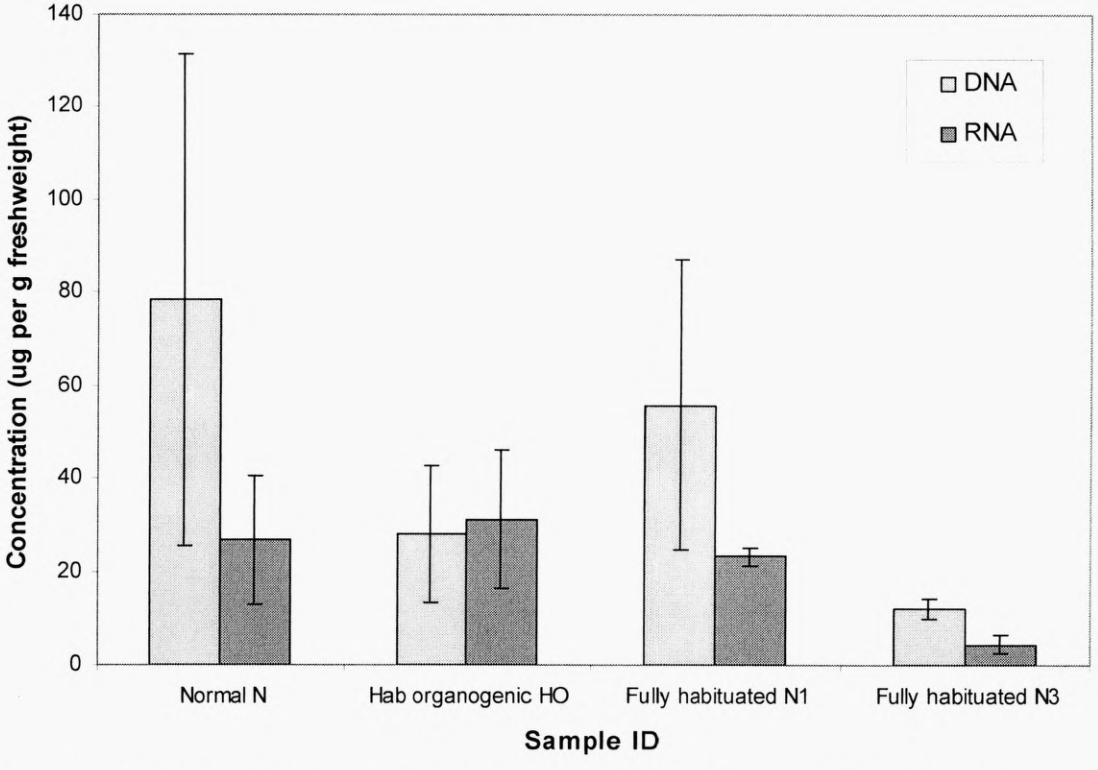


Figure 3.27: DNA and RNA concentration in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Concentration is based on µg per g of fresh weight material. Errors are derived from the standard deviations of the data series.

The data showed that there was significantly lower amount of DNA in the fully habituated cell line (N3) ($P<0.05$, $H=8.44$). The Kruskall-Wallis test was applied to statistically analyse the data series. It was also observed, from figure 3.27, that in the callus cell lines N, N1 and N3 the levels of DNA were higher than that of RNA,

except in the habituated organogenic cell line HO. In this case there was a slightly higher level of RNA than DNA, suggesting an increased turnover rate of RNA, which is commonly associated with organogenesis.

3.5.2 HPLC analysis of DNA methylation

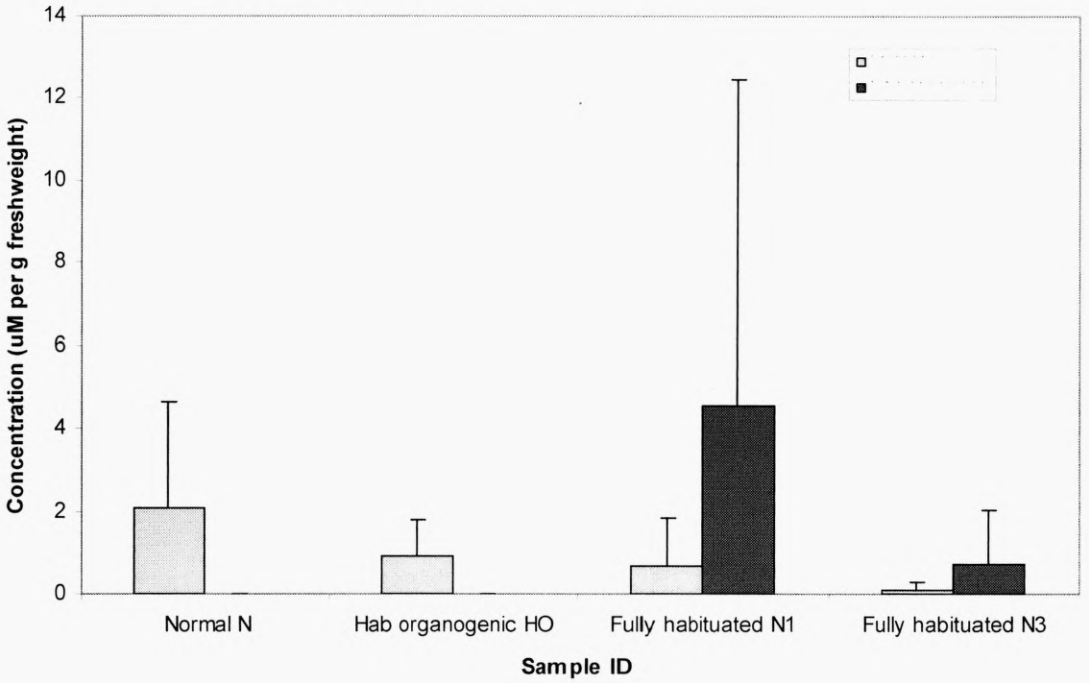


Figure 3.28: HPLC analysis of DNA methylation in *B. vulgaris* cell lines N (normal), HO (habituated organogenic) and N1, N3 (fully habituated). Data points were calculated as the means of replicate samples, where N=9. Concentration is based μg per g of fresh weight material. Errors are derived from the standard deviations of the data series. Highly variable results were observed during this assay, so standard deviations were very large.

There were no significant differences between the levels of cytosine detected in any of the four cell lines. 5-Methylcytosine was only detected in the fully habituated cell lines, however the replicate samples were highly variable due to the very low levels of DNA that were successfully extracted from the material, therefore the results could not be accurately assessed with confidence due to lack of DNA recovery.

3.6 DISCUSSION: A STUDY OF *IN VITRO* HABITUATION AND NEOPLASTIC PROGRESSION IN *B. VULGARIS*

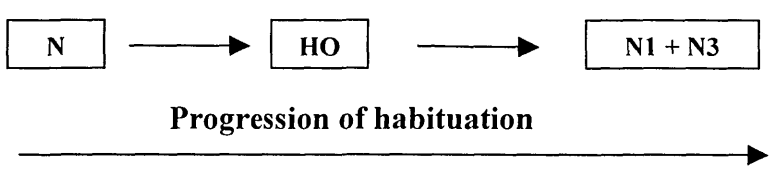
The *B. vulgaris* cell lines can be divided into three groups:

(a) "Normal", non-habituated cell line (N), which maintains dedifferentiated growth (callus) in the presence of PGRs supplemented in the medium and therefore this cell line can be classed as PGR dependent and is often used as the "control" cell line in previous studies (Hagège *et al.*, 1994).

(b) Habituated organogenic cell line (HO), which is capable of organogenesis (in the form of shoots, no roots formed) in the absence of any PGRs in the medium, therefore this cell line is PGR independent. As this cell line is capable of organogenesis it is considered only to be partially habituated as it has still maintained some regenerative capacity.

(c) Two fully habituated cell lines (N1 and N3) which have lost all regenerative capacity, are incapable of forming any organised structures such as shoots or roots, however do proliferate as completely dedifferentiated callus in the absence of PGRs, which are usually required in normal cells to maintain the dedifferentiated state, therefore N1 and N3 are also PGR independent.

Overall the four cell lines can be classed as being at differing stages of habituation progressing as follows:



3.6.1 Consideration of pro and anti-oxidant status: in vitro implications

A considerable amount of research, largely undertaken by Gaspar and his team (see Gasper *et al.* 1988-2000) has been previously conducted on this unusual and unique set of habituated *B. vulgaris* cell lines, which were originally provided for this project by Th. Gaspar. More recently, studies on the cultures include the ramifications of the findings of (Häsler, 2003) concerning early neoplastic progression in the normal *B. vulgaris* cell line. With this in mind, this discussion will first overview the previous studies and then highlight the more detailed advances of the current findings (this thesis) which, for this first time offers a greater insight into pro-oxidants, antioxidants and cellular and morphological responses of neoplastic progression in total. Clearly these studies highlight the complexity of the different components of neoplastic progression and habituation and it is essential to note that in the case of oxidative and antioxidant stress investigations their interactions occur across many levels. Therefore, this discussion will first proceed by bringing together the findings of previous researchers in the context of pro and antioxidant status *in vitro*.

3.6.2 Pro-oxidant status, cellular and morphological competence

In 1988, Crèveocour *et al.* described the fully habituated cell lines (i.e. N1 and N3) as being incapable of expanding and differentiating compared to the normal, non-habituated cell line (N). Gaspar *et al.* (1988) reported on the very low peroxidase activity in the fully habituated cell lines; peroxidase plays an active role in cell wall formation, specifically in the polymerisation of lignin monomers and the cross-linking

of cell wall polymers. Thus, they confirmed that reduced peroxidase activity was associated with the low lignin and cellulose levels in the fully habituated cell lines. Later research showed that the fully habituated cell lines lacked well differentiated plastids (Crèvecoeur *et al.,* 1992) and were also deficient in chlorophylls (Hagège *et al.,* 1992c).

Ultra-structural characteristics of the *B. vulgaris* cell lines were analysed in detail by Crèvecœur *et al.* (1992). The most apparent differences reported between the normal, non-habituated and fully habituated cell lines were observed in the vacuoles. The normal cell line had large vacuoles, normally one or two, which filled almost the whole cell. In contrast, in the fully habituated cell lines some of the cells had several, smaller vacuoles. The morphology of the mitochondria was also different in the normal and habituated cell lines; the mitochondria appeared normal in the non-habituated cell line however in the fully habituated cell line the mitochondria were reported to be elongated and dumbbell shaped. The abnormal morphology of mitochondria is frequently associated with the impacts of uncoupled energy production, electron escape from the electron transport chain and free radical production (Benson, 1990). The cell walls of the fully habituated and the normal cell lines showed apparent differences with the fully habituated cell lines displaying incomplete cell division, which is most probably caused by the lack of cellulose and lignin (Crèvecoeur *et al.,* 1992). This also supports (*as discussed above*) the implication of peroxidases being involved in cell wall synthesis and abnormal development. There were also noticeable differences (Crèvecoeur *et al.,* 1992) in the nucleus with respect to their shape and number of nucleoli; in the fully habituated cell lines the nucleus was not as well defined as in the normal cell line and this was

suggested to be as a result of an increase in the exchange surface between the nucleoplasm and the cytoplasm. The fully habituated cell lines also had multiple nucleoli whereas the normal cell lines tended to only have one (Crèvecoeur *et al.*, 1992). Hagége *et al.* (1992) noted that the shape of the nucleus in the fully habituated cell lines was abnormal and had large invaginations in both membranes of the nuclear envelope, which were not present in the normal cell line.

The organogenic tumours, such as those in cell line HO, are considered to be less advanced (in terms of neoplastic progression), as they have maintained partial totipotency compared to the cells of the fully habituated cell lines, which have lost all totipotency. Previously reported characteristics in this cell line include hyperhydric appearance of the abnormal shoot structures (also see Figure 3.3), where the shoots are described as being watery and paler than that of normal shoots and lowered lignin levels (Gaspar *et al.*, 2000). Habituated organogenic cultures have been compared to the plant equivalent of a teratomas in mammalian cells (Leshem, 1986 and Gaspar 1991). The abnormalities reported in the HO cell line are thought to result from malfunctioning meristems and previously reported morphological characteristics include abnormal shoots (elongated, wrinkled curled tips), failure to root and simultaneous necrosis of all meristematic centres (resulting in death of whole shoot clusters). Occasional deviation from shooting to callusing and "cauliflower-like" structures forming at the base of the shoots were previously reported (also see Figure 3.3), where true stems are no longer recognisable (Gaspar *et al.*, 1991) and vitrification at this stage is said to be irreversible (see Table 3.5).

Material	PGR dependency	Status of meristems	Organogenic capacity	Reversibility
Whole plant or normal shoot culture	+	Functioning well	Normal	N/A
Vitrified shoot culture	+	Functional	Shows some abnormal signs	Reversible
Severely vitrified shoot culture	_	Functional	Many abnormal characteristics	Reversible
Severely vitrified culture deviating from shoots to callus	_		Completely abnormal	Irreversible

Table 3.5: Table to show the different stages of vitrification in shoot cultures and the effects on ability to produce organic structures and the reversibility of the vitrification. Adapted from Gaspar *et al.* (1991).

3.6.3 Ethylene, polyamines and their implications for pro-oxidant status

Ethylene has been associated with the enzymes that are involved with cell wall synthesis (Roberts *et al.*, 1988). Ethylene levels and the levels of its precursor ACC were investigated in the habituated *B. vulgaris* cell lines and Hagège *et al.* (1991) reported that the levels of ethylene in these species were considerably lower in the fully habituated cell lines compared to the normal cell line. The biosynthetic pathways of ethylene and polyamines share the same precursor, S-adenosyl methionine (SAM), and so are in competition with each other. This is an important consideration in plant tissue culture responses as polyamines are antioxidants and has free radical scavenging capabilities. In contrast, the biosynthetic pathways of

ethylene and ACC synthase have free radical intermediates. These two pathways are important in neoplastic responses as confirmed by Hagége *et al.* (1994) who showed that in the fully habituated cell lines, whilst ethylene levels were extremely low the levels of accumulated polyamines were very high. The fully habituated cell lines N1 and N3 have also been shown to have altered nitrogen metabolism (Le Dily *et al.*, 1993), which has also been associated with polyamines and changes in antioxidants levels.

The habituated organogenic cell line HO has received much less attention than the fully habituated cell lines (N1 and N3). This is due to the fact that this cell line has maintained partial totipotency (shoot formation) and has not yet reached the final neoplastic endpoint seen in the fully habituated cell lines and it is a difficult line to handle. Ethylene and polyamine levels are among the few biochemical characteristics that have been examined in these cell lines (Hagège *et al.*, 1994). It was reported that the HO cell line had similarly low ethylene levels; however it did not have the same elevated levels of polyamines, suggesting that perhaps benzoic acid (an inhibitor of polyamine conversion) did not accumulate in the same way as in the fully habituated cell lines, allowing the conversion of polyamines to tetrapyrolle compounds. Another possibility is that SAM, the common precursor in the ethylene and polyamine pathway and also for DNA methylation, can take part in a C1 pathway as it is a major source of methyl groups and is used to produce compounds such as lignin, methylated esters and pectin (Hagège *et al.,* 1994). These are all involved in cell wall synthesis, therefore may be still very active since this cell line is organogenic. This has important implications for *in vitro* culture responses as these "markers" of pro- and antioxidant metabolites/hormones may be putative indicators of morphogenetic

competencies in cultures that are progressing towards a habituated and/or neoplastic state.

3.6.4 Carbohydrate metabolism and pro-oxidant and antioxidant status

Levels of sugars, sucrose, glucose and fructose were measured in *B. vulgaris* cell lines to determine if they had disturbed carbon metabolism (Bisbis *et al.*, 1993). Sucrose uptake was significantly higher in the normal cell line compared to the fully habituated cell lines over the first ten days of the subculture period. In contrast, the glucose and fructose levels accumulated more in the fully habituated cell lines as compared with the normal cell line and contrasting results prompted a further study of the activity of the enzymes that metabolise these sugars. It was discovered (Bisbis *et al.*, 1993) that the accumulation of the reducing sugars, fructose and glucose, in the fully habituated cell lines was as a result of a decrease in activity of the enzymes involved in their metabolism. This is a very important consideration in the case of oxidative stress, which is precipitated by abnormal and uncoordinated primary metabolism. However, in the case of *B. vul*garis sugar metabolism, it is important to consider that sugar accumulation is also a physiological trait of this species. The fact that this can occur *in vitro* requires further consideration particularly when autotrophic metabolism is not the standard route of metabolic energy. Indeed, differential changes in normal sugar metabolism could also be used as additional markers (e.g. the types of sugars) when evaluated in relation to pro- and antioxidant status.

3.6.5 Primary antioxidants, their activity and their implications for pro-oxidant status

The last comprehensive review of a wide range of antioxidants in these *B. vulgaris* cell lines was carried out in 1992 by Hagège *et al.* (1992b) who compared the activity of several key antioxidants such as catalase, superoxide dismutase and glutathione reductase and also determined the concentration of intracellular antioxidants glutathione and ascorbate. They compared levels of the above in the normal non-habituated cell line and the fully habituated cell lines, they reported that there were very low levels of ascorbate in both the normal and fully habituated cell lines and the levels of glutathione were not significantly different between the cell lines. Catalase levels were significantly higher in the normal cell line compared to the fully habituated cell line and SOD activity was significantly higher in the fully habituated cell line during the first ten days of the culture period after which SOD levels in the normal cell line overtook levels in the fully habituated cell line. Glutathione reductase levels were significantly higher in the fully habituated cell lines. Hagége *et al.* (1992) did not study any antioxidant levels in the habituated organogenic cell line. The low levels of catalase and peroxidase in the fully habituated cell lines (Hagège *et al.*, 1992c) will result in these cell lines having higher hydrogen peroxide levels and it is proposed that increased hydrogen peroxide causes a rapid activation of the hexose monophosphate pathways as a result of the enzymes in the glycolysis pathway being deactivated by thiol oxidation (Bisbis *et al.*, 1993). These findings will be discussed later in this chapter with respect to SOD levels in the current study.

3.6.6 *Patterns of gene expression and its association with loss of totipotency and subsequent neoplastic progression*

Tacchini *et al.* (1995) reasoned that the hormonal imbalance and loss of totipotency in the fully habituated cell lines might be caused by the activation or repression of specific genes, and therefore there would be differences in specific proteins produced in each cell line. Their work showed that the normal (N) *B. vulgaris* cell line contained five specific proteins which did not appear in the fully habituated cell lines and they found that the fully habituated cell line contained one single exclusive protein, which did not appear in the normal cell line. Further research on these proteins may help in determining the exact role they play in the habituation process.

3.6.7 *Recent evidence of spontaneous habituation in long term in vitro tissue culture*

A more recent review re-examined some of the previous cytological characteristics in the normal (non-habituated cell line) and the fully habituated cell lines (Häsler *et al.*, 2003). It was found that some of the previous characteristics, exclusive to the fully habituated cell line, were now visible in the normal cell line. Thus it may be asked, has the normal cell line undergone changes or neoplastic progression, which has lead to the formation of these new abnormalities previously only seen in the fully habituated cell lines? Häsler *et al.* (2003) reported on the appearance of buds on the surface on the normal cell line and incomplete cell walls (perhaps as a result of decreased lignin or cellulose) and they suggested that the normal cell line is entering spontaneous habituation and may be undergoing neoplastic progression.

3.7 INTEGRATION OF RECENT ADVANCES WITH PREVIOUS STUDIES OF HABITUATION.

The following section discusses the findings from the current studies in respect to the morphological, cytological and antioxidant properties of the habituated *B. vulgaris* cell lines and considers the effect of these on the pro-oxidant status of the cell lines.

3.7.1 Current morphological and cytological characteristics of the habituated B. vulgaris cultures

Morphologically, the normal, non-habituated cell line appears to resemble an ordinary callus culture, defined by its anarchic proliferation of dedifferentiated cells in the presence of PGRs. The cells are compact and dry compared to the watery cells seen in the fully habituated cell lines (N1 and N3). This cell line (N) may have slightly reduced chlorophyll levels as the callus was pale green in colour (see Figure 3.2), which may indicate that this cell line has undergone a decrease in chlorophyll levels possibly due to a decrease in functional chloroplasts caused by *in vitro* ageing and the associated oxidative stress. The normal cell line (N), like most other callus culture, requires plant growth regulators in the culture medium to maintain dedifferentiated growth and is not reported to be showing signs of loss of auxin and cytokinin requirement.

Cell line N is non-embryogenic; the loss of embryogenic potential is not unusual in callus cultures particularly in cultures maintained dedifferentiated in *in vitro* tissue

culture for long periods of time (Benson *et al.*, 1992). During the first subcultures after its initial callus initiation in 1979, this cell line was capable of regenerating structured organs in the appropriate medium (De Greef and Jacobs, 1979). However, it became non-embryogenic very quickly and although several attempts have been made to regenerate organs using various mixtures of cytokinins and auxins, organised structures have never been formed (Gaspar *et al.*, 2000). This cell line, as previously mentioned, can be defined by its anarchic proliferation of dedifferentiated cells and this lack of organised structures and organs can resemble tumours, therefore this normal callus can be considered a primitive neoplastic growth (Gaspar *et al.*, 2000). In previous studies, this cell line (N) has often been used as a control to compare against the habituated organogenic (HO) and fully habituated cell lines (N1 and N3).

The recent cytological data collected by Häsler *et al* (2003) compares the normal (N) and fully habituated cell lines (N1 and N3) and has uncovered some significant cytological changes in the normal cell line (N), which would suggest that this "normal" cell line may have entered neoplastic progressions. This cell line has not reached the same extent of habituation as the fully habituated cell lines (which are thought to have reached a terminal progression), as there are still several cytological differences, suggesting that if this cell line is entering neoplastic progressions, it is in the very early stages. The cytological changes in the normal cell line, which were only previously observed in the fully habituated cell lines (N1 and N3) include cell surface protrusions, incomplete cell walls and a level of starch in plastids that were previously higher in the fully habituated cell lines and are now higher in the normal cell line (Häsler *et al.*, 2003). Some of the observations reported by Häsler *et al* (2003) were also observed in this present study, in particular signs of incomplete cell

wall and possible surface protrusions (see Figure 3.4). Therefore, overall, some of the cytological data collected during this study supports the previous findings suggesting that this, so called, normal cell line may spontaneously have entered neoplastic progression possibly as a direct result of *in vitro* aging.

The habituated organogenic cell line (HO) is an intermediate habituated line, which has undergone some neoplastic steps and become habituated against plant growth hormones auxins and cytokinins and also lost the ability to root (see Figure 3.2). The unique characteristic of this cell line is that it is still capable of regenerating organogenic structures in the absence of any plant growth hormones; however, the shoot structures that are formed are far from normal and show several morphological abnormalities including translucent, hyperhydric watery and wrinkled shoots with curled tips (see Figures 3.2 and 3.3). Often during subculture whole clumps of shoots decay and turn brown. This is a common sign of malfunctioning meristems, where all the meristem apices undergo necrosis causing death of the whole shoot cluster (Gaspar *et al.*, 2000). Abnormal cauliflower-like masses were formed at the bases of some of the meristematic shoot structures (see Figure 3.2) and completely dedifferentiated material was produced at some of the bases of the shoot structures (see Figure 3.2), where the meristems malfunctioned and could no longer form organised structures giving rise to completely dedifferentiated white callus.

This cell line has lost the ability to form roots, therefore has partially lost organogenic potential. It has also been suggested previously that this cell line was capable of producing roots on occasion, however throughout this study there was no indication of any root formation which may suggest that this cell line may have lost, in terms of

root potential, more organogenic potential. The abnormalities observed in the shoot structures have been reported previously by Gaspar *et al.* (2000) and Kevers *et al.* (1981), therefore there appeared to be little further change in the morphology of this habituated organogenic cell line (HO). Functional meristems were observed in the cells using a FDA staining technique, which showed the highest esterase activity towards the shoot apex, representative of meristemic centres (see Figure 3.4). Individual cells were elongated and had a similar shape to the cells in the normal cell line (see Figure 3.4).

The habituated cell lines (N1 and N3) were initiated from the dedifferentiated callus material that was formed at the base of the organogenic cell line (HO) caused by malfunctioning meristems (De Greef and Jacobs, 1979). As these cells were derived from cell line HO, these callus cell lines (N1 and N3) are also both habituated and, unlike those from which they were initiated, are completely dedifferentiated and incapable of forming organised structures. They proliferate in a chaotic fashion in the absence of any PGRs, which are normally required for dedifferentiated growth in *in vitro* tissue culture (see Figure 3.2). These fully habituated cell lines (N1 and N3) are thought to have reached the endpoint of neoplastic progression, where they have lost all totipotency and are at a point of no return, at this stage cells can be considered to be truly cancerous (i.e. fully habituated).

Cell lines N1 and N3 differ in a number of ways from a normal callus cell line. They have half the subculture period compared to cell line N, due to extensive cell necrosis after day 12-14 (drying, shrinking and browning was observed). They lack chlorophyll (yellow colouration suggests that there may still be some carotene

pigments expressed as well as chlorophyll moieties) and therefore the resulting callus is yellowish/white (see Figure 3.2). The fully habituated cell lines (N1 and N3) are sensitive to light, probably as a result of the lack of chlorophyll pigments which would normally remove any excess ROS produced from light energy. Therefore, to prevent subsequent necrosis it is necessary to keep these cultures under a layer of gauze. Growth analysis demonstrated that the fresh weight gain in the fully habituated cell lines (N1 and N3) was half of that of the normal cell line; also the fully habituated cell lines appeared watery, very friable and lack cell-cell adhesion compared to that of the normal callus (N).

The fully habituated cell lines have also been shown to have several irregular cytological and biochemical characteristics including imperfect cell wall formation and incomplete chloroplast and mitochondria differentiation (Crèvecoeur *et al.*, 1992). The cytological analysis during this study showed some significant differences between the normal cell line and the fully habituated cell lines. The former were elongated (see Figure 3.4) compared to the fully habituated cell lines where the cells were considerably smaller and more spherical (see Figure 3.4). Using a light microscope at X 100 magnification showed signs of membrane damage and cell contents being released from the cell (see Figure 3.4). Note that in this context FDA staining was used to help elucidate the morphological structure rather than cell viability as the cells were taken from growing cultures. The level of esterase activity (indicated by highly fluorescent green areas) appeared to be much higher in the fully habituated cell lines, which are chaotic, rapidly dividing small cells (see Figure 3.5). There were no significant cytological changes in this fully habituated cell line identified during this study or in data collected by Häsler *et al.* (2003) compared to

the data that was reported by Crèvecœur *et al.* (1992) suggesting again that these cell lines appear to have reached their terminal neoplastic progression.

3.7.2 Antioxidants, as putative "markers" of morphology and neoplastic progression

The study of the biochemical characteristics of the *B. vulgaris* cell lines included the determination of the activity of key antioxidants including catalase, peroxidase, superoxide dismutase, glutathione reductase, glutathione S-transferase, glutathione, ascorbate and sulphydryl groups. The levels of hydroxyl radical and hydrogen peroxide were measured and, finally, lipid peroxidation products lipid hydroperoxides, conjugated dienes, malondialdehyde, 4-hydroxyalkenals and Schiff's bases were determined in order to compare the extent of ROS damage in each of the cell lines. By collecting this data, the pro-oxidant and antioxidant potential of the lines can be determined giving an overview of the effectiveness of the antioxidant systems in place to mitigate against oxidative stress associated with *in vitro* cell ageing and cancer.

Summary of enzymatic and non-enzymatic antioxidant profiles in the four cell lines of the *B. vulgaris* cultures

Antioxidant	**Normal (N)**	**Habituated organogenic (HO)**	**Fully Habituated (N1)**	**Fully Habituated (N3)**
Catalase Enzyme activity per $mgprot^{-1}$	0.002359 ^{c}	0.00141 ^{c}	0.000718 ^{c}	0.000985 ^{c}
Peroxidase Enzyme activity per $mgprot^{-1}$	0.00759	0.02324 $^{\uparrow b}$	0.006543	0.016370
Cu,Zn – SOD Enzyme activity per $mgprot^{-1}$	0.0 ^{c}	0.0 ^{c}	0.0 ^{c}	0.0 ^{c}
Glutathione reductase Enzyme activity per $mgprot^{-1}$	0.0322 ^{c}	0.0335 ^{c}	0.0383 ^{c}	0.0475 ^{c}
Glutathione S-transferase Enzyme activity per $mgprot^{-1}$	0.00023	0.000352	0.000937 $^{\uparrow a}$	0.000794 $^{\uparrow a}$
Total glutathione mM per gfw^{-1}	1.309 ^{c}	1.0751 ^{c}	0.8327 ^{c}	0.6388 ^{c}
Oxidised glutathione mM per gfw^{-1}	0.3740 ^{c}	0.3806 ^{c}	0.5018 ^{c}	0.5086 ^{c}
Total ascorbate µM per gfw^{-1}	32.700	36.529	24.612 $^{\downarrow a}$	22.906 $^{\downarrow a}$
Reduced ascorbate µM per gfw^{-1}	11.301	12.134	7.776 $^{\downarrow b}$	11.055
Protein-bound Sulphydryl µM per gfw^{-1}	4.446	8.884	0.0753 $^{\downarrow a}$	-0.08393 $^{\downarrow a}$

Table 3.6: Summary of the absolute values obtained from the biochemical analyses of the key antioxidants in the *B. vulgaris* cell lines during this study. Statistical differences between the cell lines are marked as follows: ↑a or ↓a = very highly significant, ↑b or ↓b = significant, c = no significant differences, the direction of the arrow denotes whether it is a higher or lower value.

3.7.2.1 Enzymatic oxidants, superoxide dismutase, catalase and peroxidase

Superoxide dismutase is the enzyme responsible for the dismutation of superoxide radicals, which are the first radical species to be formed after electrons leak from the electron transport chain (see Figure 3.29). In plants, Cu,Zn-SOD is located in the chloroplast, cytosol and peroxisomes and Mn-SOD is located in the mitochondria. As the majority of Cu,Zn-SOD is found in chloroplasts; cell lines that experience a decrease in the number of functional chloroplasts may have a corresponding decrease in Cu,Zn-SOD activity. The colour of the N cell line callus was a pale green, which

may suggest a loss in chlorophyll and may be indicative of a loss of functional chloroplast numbers. However as the number of functional chloroplasts were not determined during this study it is difficult to confirm this; also these cells are grown heterotrophically. Analysis showed that no Cu,Zn-SOD activity was detected in any of the four *B. vulgaris* cell lines. This data suggests that a connection between Cu,Zn-SOD activity and levels of chlorophyll in the *B. vulgaris* cell lines can be eliminated as, although there was no chlorophyll in the fully habituated cell lines, it was still be present in the normal cell line.

The complete absence of certain types of SOD, in particular manganese containing SODs has been shown to be a characteristic of several different cancerous (Oberley and Buettner, 1979) and ageing mammalian cell lines (Antier *et al.,* 2004) and in a few cases the absence of copper, zinc containing SODs has been reported (Oberley and Buettner, 1979). Importantly, the loss of SOD activity in cancerous and ageing plant cell lines has not been previously reported, therefore could it be the case that these cell lines have lost Cu,Zn-superoxide dismutase activity as a result of habituation (cancer) or *in vitro* ageing? This question may be more effectively answered using western blotting to analyse specific proteins, which would allow a more accurate measurement of each of the metal ion forms of SOD, their activity and their cellular location. This is recommended in future studies.

Hagége *et al.* (1992) reported on total SOD activity in the *B. vulgaris* cell lines, i.e. Mn, Cu-Zn and Fe containing SODs. They found that there was a pattern in activity in both the normal cell line and the fully habituated cell line. During the initial days of the subculture in both cell lines there was an immediate peak in SOD activity

(higher in the fully habituated cell line), followed by a slight drop then a steady increase in the normal cell line. However, in the fully habituated cell lines the activity simply tailed off after the initial peak and fell to much lower levels than that of the normal cell line. Their report suggests that the fully habituated cell line is able to respond to high superoxide levels at the beginning of the sub-culture period, however could not maintain the level of protection as well as the normal cell line.

The decrease in activity in the fully habituated cell line may be connected to the lack of chloroplast maturation observed in these cell lines (Hagège *et al.*, 1992c). It may have been advantageous to measure the activity of manganese containing superoxide dismutases to confirm whether these cell lines had lost all types of superoxide dismutase activity. However, the absence of the most abundant SOD (Cu,Zn-SOD) in all these cell lines is a significant finding. Perhaps the gene for this enzyme has been affected at different levels from gene expression to translation. Also this may involve differences in constitutive and induced expression related to pro-oxidant status. Or, no SOD detected because it is used very quickly due to high levels of superoxide radicals and the assay components may have a lower affinity than the removal of superoxide radicals. However, this is less likely based on other findings (Benson *et al.*, 1992). The results would appear to suggest that there is no Cu,Zn-SOD activity. However future studies and the gene expression level would be necessary to determine whether this cell line has lost global SOD expression and activity.

Most importantly a lack of/or deactivation of SOD will lead to an imbalance in normal ROS levels and will have a down-stream effect on the way the cell undertakes detoxification. A decrease in SOD activity will cause a decline in the amount of

hydrogen peroxide produced and a corresponding increase in superoxide radicals. As hydrogen peroxide is increasingly thought to play a role as a signalling molecule during the growth phases in plants (Kairong *et al.*, 2002), a reduction in hydrogen peroxide levels may have serious implications in plant development. However, three of these cell lines are non-embryogenic and the fully habituated cell lines have lost all totipotency therefore it is doubtful that any hydrogen peroxide signalling occurs. The only cell line that may be using hydrogen peroxide as a signalling molecule would be the habituated organogenic cell line (HO) and this cell line has several abnormalities, which may be caused by changes in cell signalling activities.

A decrease in hydrogen peroxide production (caused by a lack of superoxide dismutase) may, on the other hand, be beneficial to the cell particularly in aged tissue as a means of limiting ROS formation. Excess hydrogen peroxide can take part in the Haber-Weiss reaction which gives rise to hydroxyl radicals, which are, due to their high reactivity more damaging than superoxide radicals (see Figure 3.29). Therefore, is it possible that the decrease in SOD activity is an activated defence mechanism designed to reduce the levels of hydroxyl radical formed. Superoxide radicals, although they are a reactive species, are not as harmful as the hydroxyl radical. Superoxide radicals can also be removed by other cellular antioxidants (aside from SOD) that include glutathione and ascorbate, which will detoxify superoxide radicals without the production of hydrogen peroxide. However, these can only operate as effective antioxidants when the ratio of reduced glutathione or ascorbate to oxidised glutathione or ascorbate is high. When the ratio of oxidised oxidants such as GSSG and DHASC is higher than their reduced counter parts then the antioxidant / pro-oxidant equilibrium is likely to shift towards pro-oxidation, where pro-oxidant

reactions (reactions involving ROS) can be promoted and superoxide radicals are highly likely to take part in further ROS reactions before they can be detoxified.

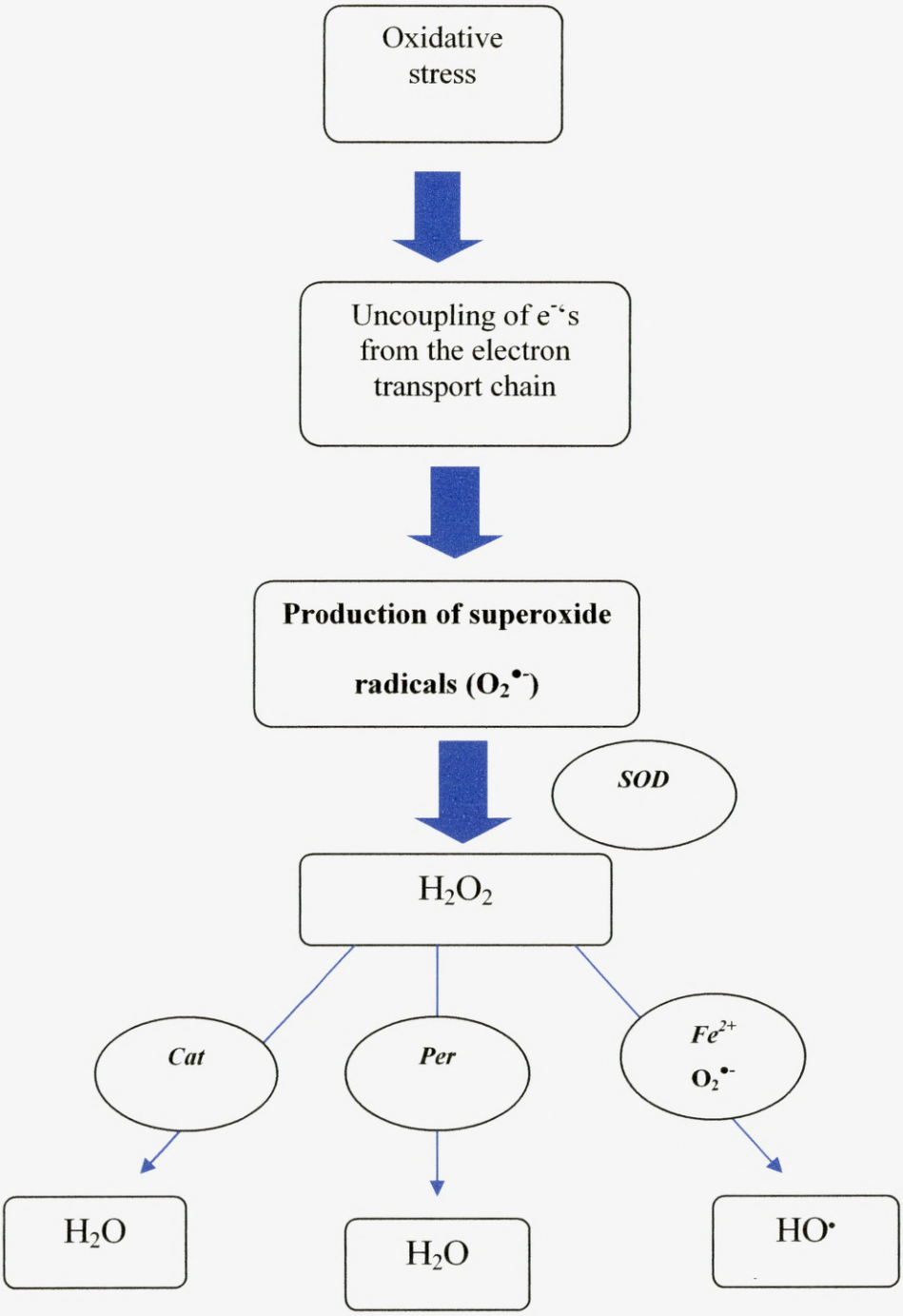


Figure 3.29: Schematic diagram of the initial stages of oxidative stress beginning from the uncoupling of electrons from the electron transport chain, as a result of a metabolic disturbance, leading to the formation of superoxide radicals. Superoxide radicals ($O_2^{\bullet -}$) are then detoxified by superoxide dismutase (SOD) to produce hydrogen peroxide (H_2O_2), which is then either decomposed by catalase (cat) or peroxidase (Per) or can take part in Fenton/Haber-Weiss reactions in the presence of $O_2^{\bullet -}$ and Fe^{2+} to give the highly destructive hydroxyl radical ($OH^{\bullet}$).

Peroxidase and catalase are the two main enzymes involved in the detoxification of hydrogen peroxide (see Figure 3.29). Peroxidases have often been referred to as markers of morphogenesis (Faivre-Rampant *et al.*, 1998) and have been shown to be much lower in cell lines which have lost embryogenic potential (Halliwell, 1981c) and increased peroxidase activity is often associated with cells that contain increased lignin, which is involved in cell wall synthesis. Peroxidase activity has been shown to be very low in habituated carrot callus (Joersbo *et al.,* 1989) and was also very low in non-embryogenic lettuce cultures (Zhou *et al.,* 1992). Peroxidase activity was significantly higher in the organogenic (HO) cell line (see Figure 3.8), which is capable of generating shoot structures, and is therefore actively producing lignin, a suitable substrate for peroxidase. In the other three cell lines, which were all callus and are known to contain very little lignin, peroxidase activity was very low. One of the sets of replicates from the habituated cell lines (N3) exhibited unusually high peroxidase activity. This set of anomalous results were derived from the same plate but were consistent and reproducible and the standard data collected for this assay had a line of fit consistently above 90%. Therefore, it was accepted that this one plate exhibited unusually high peroxidase activity, for reasons unknown. The rest of the replicates collected from the other plates for this cell line exhibited similar activity levels to the other fully habituated cell line (N1).

Catalase activity has been previously correlated with the embryogenic potential of the *in vitro* culture. In rice cell suspensions catalase activity was significantly higher in the embryogenic cell line compared to that of cell lines that had lost embryogenic potential or cell lines in the process of losing the potential (Benson *et al.*, 1992). Other studies have demonstrated that catalase activity is very low in rapidly

developing tissues and since hydrogen peroxide has been recognised as a signalling molecule in plants (Kairong, 1999, Neill *et al.,* 2002) it appears to be particularly involved during somatic embryogenesis. Kairong *et al.* (1999) showed that by manipulating the levels of hydrogen peroxide by inhibiting catalase activity they could promote somatic embryogenesis. They monitored the levels of catalase in *Lycium barbarum* L. *in vitro* tissue cultures during somatic embryogenesis, and found that during the callus formation stage the levels of catalase were high. Then during early differentiation, levels of catalase rapidly declined for approximately four days after which catalase activity gradually increased to the level that it was in the callus. Therefore it appeared that a reduction in catalase activity lead to an increase in hydrogen peroxide, which in turn stimulated the embryogenesis.

In grape vine cultures, catalase profiles were measured throughout the process of callus induction. Catalase activity was measured in the explant material and then in the callus material that resulted after treatment with callus induction PGRs. In the explant (embryogenic material) sample, catalase activity was very low; however in the callus material catalase activity increased dramatically (Benson and Roubelakis-Angelakis, 1994). These results suggest that in morphogenic cell lines hydrogen peroxide concentration is high whilst in callus material the hydrogen peroxide levels are low during the first four days of callus induction. Is hydrogen peroxide therefore required to facilitate organ formation? Considerable research now suggests that hydrogen peroxide promotion of embryogenesis is definitively concentration dependent (Kairong, 1999). Catalase activity declined dramatically in i*n vitro* tissue cultures of grape vine over time (2 months) (Benson and Roubelakis-Angelakis, 1994) perhaps due to a disruption in enzyme regulation or ROS deactivation, as during most

antioxidant studies on *in vitro* cultures the decrease in antioxidant potential has been accompanied by an increase in ROS and lipid peroxidation products. Whether the loss of catalase activity in long-term *in vitro* tissue culture contributes to the progressive loss of embryogenic potential or catalase reduction is a consequence of differentiation has not yet been identified and this area would benefit from further research.

Overall, it would appear that hydrogen peroxide (regulated by catalase) does play a role in embryogenesis occurring in explants and newly differentiated callus. However, the contradicting observations suggest that catalase can only play an active role for a limited period of time during *in vitro* manipulation of plants and there are also several reports suggesting the age of the callus has a role (Rikans and Hornbrook, 1997, Azhar *et al.,* 1995). The results from this present study showed that all the *B. vulgaris* cell lines had extremely low catalase activity in all of the four cell lines (see Figure 3.7). As these lines are considered to have either lost embryogenic potential (N, N1 and N3) or are losing embryogenic potential (HO) and are all thought to be aged the results suggest that ageing and possibly loss of embryogenic potential may be connected with the loss of catalase activity. These results support the findings of Benson *et al.* (1992) and Rikans *et al.* (1997).

Catalase was measured in the normal callus cell line by Hagège *et al.* (1992) who found activity was significantly higher in the normal cell line. Current work indicates that the normal cell line has lost more catalase activity since the last study in 1992, perhaps as a result of further ageing. However in these cell lines, which are considerably aged, an increase in ROS caused by long-term *in vitro* culturing may

also play a large part in the decrease of catalase activity by ROS mediated enzyme deactivation, leading to further disruption in metabolic processes.

3.7.2.2 Impacts of the glutathione-ascorbate redox cycle: antioxidant protection and detoxification in *in vitro* cultures

Glutathione has a facile electron-donating capacity due to its sulphydryl group (SH) and is a very important water-phase non-enzymatic antioxidant and is an essential co-factor for antioxidant enzymes such as glutathione reductase and glutathione S-transferase. Its high electron donor capacity combined with its high intracellular concentration gives glutathione excellent reducing power and hence an excellent antioxidant. Glutathione is used to regulate the highly complex thiol exchange systems, which are required to regulate levels of low molecular weight (non-protein bound) thiols, re-reduction of –SH protein bound thiols, the recycling of ascorbate and the re-reduction of highly complex cellular –SH proteins.

The reducing power of glutathione is a measure of its free radical scavenging ability (i.e sulphydryl donating capacity) and consequently its effectiveness as an antioxidant. For glutathione to function as an effective antioxidant it needs a reducing environment. In normal healthy cells glutathione exists almost 90% in its reduced form, which helps drive antioxidant reactions (Carvalho and Amâncio, 2002). Glutathione is thought to be very responsive towards oxidative stress and can increase the rate of ROS detoxification when the levels of ROS increase. The percentage of reduced glutathione was calculated for the *B. vulgaris* cell lines to determine whether they were below the normal 90% reduced level (see Table 3.7).

B. vulgaris Cell line	Percentage reduced glutathione
Normal N	71%
Habituated organogenic HO	65%
Fully habituated N1	40%
Fully habituated N3	20%

Table 3.7: Percentages of reduced glutathione, calculated from determining the total and the oxidised GSSG levels. The resulting reduced (GSH) concentration was then expressed as a percentage of the total GSH content.

There was a significant decrease in the percentage of reduced glutathione levels in these cell lines and the order of reduced glutathione ran in parallel with the progression of habituation. The normal (N) cell line had the highest percentage of reduced glutathione but, below 90%. This may indicate that it is experiencing an increase in oxidative stress resulting in depleted glutathione levels, probably induced by *in vitro* ageing or spontaneous habituation as suggested by Häsler *et al.* (2003).

Although there was an overall decrease in the levels of reduced glutathione (GSH), there was not a corresponding significant increase in oxidised glutathione (GSSG). This suggests that rather than a shift in the ratio there is actually a decrease in available reduced glutathione, possibly as a result of GS-conjugate formation, via glutathione S-transferase. Depletion of glutathione is caused by an increased rate of detoxification, without a corresponding increase in glutathione production, suggesting therefore that the rate of synthesis cannot keep pace with the rate of ROS detoxification.

A high ratio of glutathione is essential to maintain the overall redox potential, which controls the regulation of many metabolic processes and acts as a biological "buffer". Changes to the overall redox potential can lead to the uncoupling of essential reactions and result in loss of redox control that may have detrimental effects on a number of cellular processes. As the ratio decreases, oxidative reactions may be promoted giving rise to an overall increase in ROS reactions and the production of secondary radical species. The fully habituated cell lines, N1 and N3, both have a ratio of reduced glutathione below 50%, which points towards a severe disruption in redox regulation shifting the normal antioxidant / pro-oxidant equilibrium towards the pro-oxidant side. This results in an overall increase in the level of ROS, promoting oxidative ROS reactions, and leading to further cellular damage, such as lipid peroxidation, not to mention disturbed primary metabolism. All of these factors will also contribute to the abnormal cytology and morphology observed in these cell lines.

Ascorbate, existing mainly in its reduced form (>90%), is reported to play a role in plant development during early growth and cell dedifferentiation. Then during the latter stages of development there is a shift towards the oxidised form (DHASC), which is thought to act as a source of reduced ascorbate (ASC) when required, oxidised ascorbate can be re-reduced *via* the Halliwell-Asada cycle to form reduced ascorbate (ASC) (Carvalho and Amâncio, 2002). Ascorbate, unlike glutathione, does not respond as sensitively to elevated ROS levels and it has been suggested that ascorbate only plays a very active role on ROS detoxification during growth and developmental stages (Carvalho and Amâncio, 2002). Ascorbate may also use its redox potential to initiate certain developmental reactions. The percentage of reduced ascorbate was determined for the four *B. vulgaris* cell lines and is presented below

(see Table 3.8). Taking these factors into account and the fact that aged and morphogenetically comprised cultures were under study changes in the dynamics of ascorbate cycling may not be apparent.

***B. vulgaris* cell line**	**Percentage reduced ascorbate**
Normal N	34%
Habituated organogenic HO	33%
Fully habituated N1	32%
Fully habituated N3	48%

Table 3.8: Percentage of reduced ascorbate calculated from measurement of the total and reduced levels of ascorbate.

In support of this, the percentage of reduced ascorbate was low for all four *B. vulgaris* cell lines and there were no significant differences between the cell lines. Levels of accumulated DHASC (oxidised ascorbate) or stored ASC (reduced ascorbate) were significantly lower in the two fully habituated cell lines (N1 and N3). However it may be interesting to consider that if DHASC is allowed to accumulate in the cells it can act as an electron acceptor in protein-bound sulphydryl oxidation reactions (Szarka *et al.,* 2002). Therefore, the accumulation of DHASC can promote the oxidation of sulphydryl groups in a pro-oxidant environment. In the fully habituated cell lines the protein-bound sulphydryl groups were almost completely depleted, but still relatively high in the normal cell line, suggesting that DHASC is not the only or main cause of reduced protein-bound –SH groups.

During oxidative stress, the ascorbate recycling process is dependent on the availability of the substrates that take part in the Halliwell-Asada cycle; these include reduced glutathione (GSH) and NADPH, both of which take part in a number of other

antioxidant pathways. From the data it is also observed that glutathione levels are significantly reduced in all the cell lines, which, importantly, will have a direct effect on the ability of ascorbate to act as an efficient antioxidant. The observed reduction of the ascorbate and particularly the glutathione ratios will promote lipid peroxidation and the production of secondary products such as the highly toxic aldehydic compounds 4-hydroxyalkenals and malondialdehyde. In this scenario, GSH metabolism and detoxification also becomes important and will now be considered.

Glutathione S-transferase catalyses the conjugation of glutathione to toxic compounds that enter the cell, which enables them to be removed via glutathione transporter pumps. Furthermore, an increase in lipid peroxidation and the production of toxic compounds is often associated with an increase in the expression of glutathione S-transferase to deal with their removal. In the fully habituated cell lines (N1 and N3) the level of glutathione S-transferase activity was very significantly higher compared to the activity in the habituated organogenic (HO) and normal (N) cell lines (see Figure 3.19). Increased glutathione S-transferase expression is linked to increased 4-HNE and MDA production (Fukuda *et al.,* 1997), which is a direct result of increased lipid peroxidation. Therefore, in the fully habituated cell lines (N1 and N3) there is a very high probability that lipid peroxidation will be significantly higher in these cell lines. An increase in glutathione S-transferase (GST) activity in the fully habituated cell lines will also contribute further to the depletion of reduced glutathione. With a significant decrease in available reduced glutathione it might therefore have been expected that glutathione reductase activity would be increased to compensate for the GSH depletion. However, there were no significant differences in activity in the four *B. vulgaris* cell lines suggesting that the activity of glutathione reductase does not

respond directly to oxidative stress in these particular systems. Since glutathione reductase activity can be inhibited by glutathione conjugates, formed as a result of the enzymatic conjugation of toxic products to glutathione, *via* GST, this may explain the lower than expected glutathione reductase activity and its apparent inability to respond to the decreased glutathione ratio.

Glutathione is central to many reactions that are involved in maintaining the redox potential in cells and it appears that the redox potential is decided by the ability of the cells to maintain demand as a result of increased ROS reactions. Glutathione production depends on the rate of its own synthesis (i.e. it is self-regulatory), which means that it is dependent on the activity of the two enzymes and substrates involved in the reaction, which may also be prone to ROS deactivation. The process of recycling glutathione also adds to the reduced glutathione pool, however again this reaction is dependent on the activity of glutathione reductase, which may also be prone to ROS deactivation, since this reaction is also dependent on the availability of NADPH, which acts as an electron donor. During oxidative stress, NADPH can also act as a direct antioxidant (Kirsch and De Grout, 2001), which may reduce the overall recycling processes. Glutathione takes part in a number of reactions including; direct antioxidant, takes part in the Halliwell-Asada reaction to recycle ascorbate, and glutathione S-transferase reactions. It is also involved in replacing sulphydryl groups on proteins after their oxidation and takes part in many proton donor reactions, figure 3.30) shows an overview of some of the different reactions in which glutathione participates.

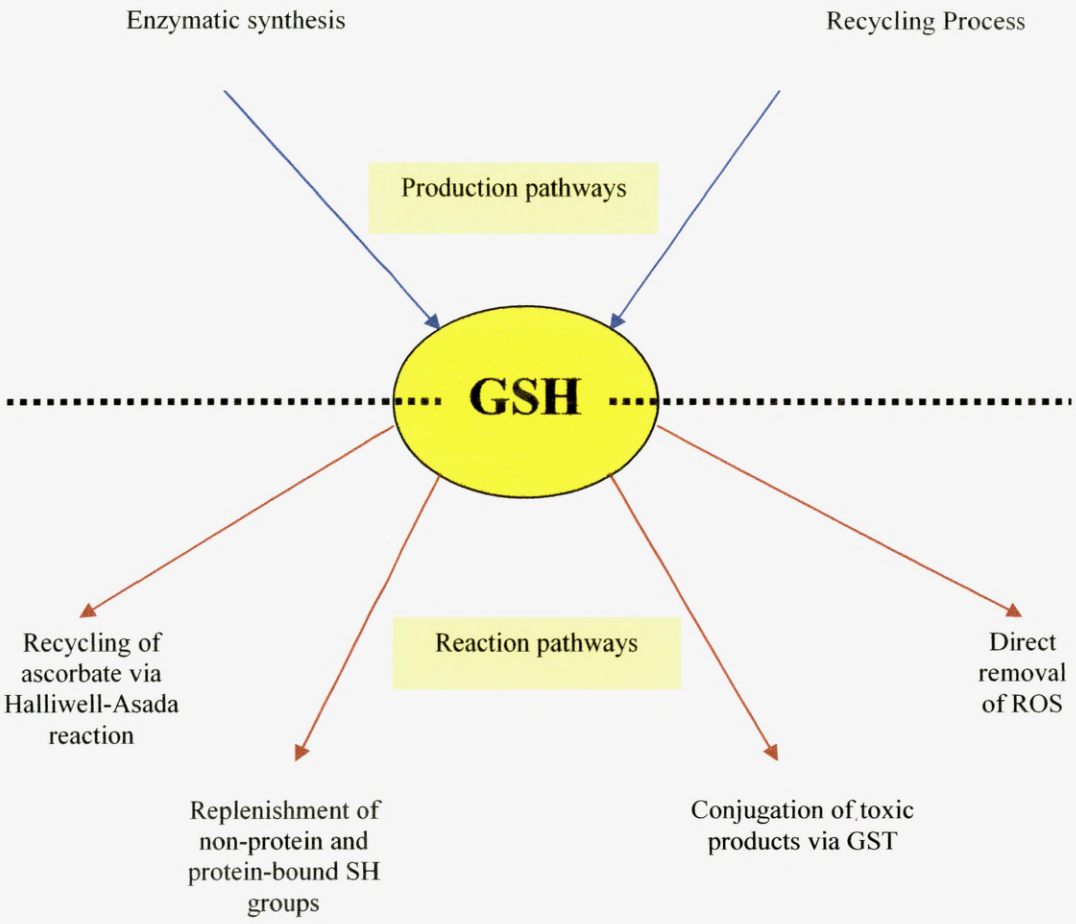


Figure 3.30: Antioxidant reactions of reduced glutathione. The diagram is divided into two sections, with the top half showing the methods of glutathione generation and the bottom half showing the antioxidant reactions of glutathione.

Glutathione conjugates can often be toxic and can sometimes be more lethal than the original product however they must be conjugated to glutathione in order for them to be expelled from the cell through glutathione pumps, but if the pumps are not functioning correctly then their accumulation can initiate further ROS reactions. Glutathione is involved in so many cellular and detoxification processes, that a decrease in its effectiveness as an antioxidant is likely to be caused by its increased demand in a combination of all or a number of the reaction pathways above.

Finally, it is important to consider GSH cycling and reactions in terms of membrane stability. Transport across cellular membranes is essential in maintaining the redox potential and effective transport is dependent on the functionality of the membrane bound enzymes which facilitate glutathione pumps, which again are prone to ROS induced damage. Defective glutathione pumps will exacerbate the accumulation of oxidised glutathione and glutathione conjugates that may not be transported out of the cell efficiently. Accumulated oxidised glutathione (GSSG) can lead to the formation of thiyl radicals ($GS^{\bullet}$) which can lead to further free radical reactions and the initiation of further lipid peroxidation. Thus, sulphydryl groups, although not considered an antioxidant, do provide the cell membrane with a degree of protection against ROS attack and subsequent lipid peroxidation. It is possible that this occurs in a sacrificial way, where the oxidation of membrane bound SH groups prevents hydrogen abstraction directly from the lipid backbone, which can quickly lead to lipid peroxidation. The oxidation of sulphydryl groups, however, can also have detrimental effects such as changes in membrane permeability and ionic imbalances (Chevrier, 1988), so it is important that the –SH groups are replaced quickly to prevent any further ROS attack. Sulphydryl groups can therefore be considered as a barrier against membrane lipid peroxidation. In a broader context, sulphydryl groups are also attached to certain enzymes, often forming the active site and their non-sacrificial oxidation, in this context, can lead to enzyme inactivation. Overall it is essential to replace –SH groups as rapidly as possible to maintain enzyme activity and to protect proteins and membranes.

In the fully habituated cell lines (N1 and N3), the level of protein-bound sulphydryl groups were completely depleted, which suggests one or a combination of the following:

(a) Enzymes involved in sulphydryl replacement have been deactivated;

(b) The redox potential of these two cell lines does not favour the replacement reaction;

(c) Limited availability of reduced glutathione delays the replacement of sulphydryl groups and overall the ROS attack rate is quicker than the –SH replacement rate.

3.7.2.3 Consequences of antioxidant compromise: Reactive oxygen species (ROS)

This part of the study involved an application of various assays to assess ROS. The detection of hydrogen peroxide in the *B. vulgaris* cell lines was unsuccessful using the chosen simple colourmetric assay. The assay was effectively validated and was highly reproducible for standard samples, with a line of fit > 90%, however hydrogen peroxide was not detected any in any of the cell lines. There are three possible reasons for the lack of hydrogen peroxide detection: (1) the range of detection is not sensitive enough for these particular cell lines (50-200μL 30% H_2O_2 per mL); (2) due to the oxidative environment any hydrogen peroxide present immediately takes part in the Haber-Weiss reactions (see section 1.9.3), where hydrogen peroxide in the presence of iron and superoxide radicals produces the highly reactive hydroxyl radicals; (3) there may be no hydrogen peroxide present perhaps due to the lack of SOD activity.

In future studies it may be prudent to examine other assays. There are a number of methods capable of measuring hydrogen peroxide, and since hydrogen peroxide is more stable than radical oxidants it has a significantly longer half-life. One such assay involves the horseradish peroxidase catalysed oxidation of fluorescent scopiletin. However, the major disadvantage of this method is that hydrogen peroxide is quantified by the decrease in the fluorescence in scopiletin, which means that often the fluorescence levels are very low and significant amplification is required, which leads to high background levels leading to decreased accuracy (Corbett, 1989). Mohanty *et al.* (1997) reported on an adapted version of this method utilising a microplate format and the novel use of a substrate N-acetyl-3, 7-dihydroxyphenoxaine, a non-fluorescent compound that becomes highly fluorescent of oxidation by H_2O_2 catalysed by horseradish peroxidase. This method was successfully used in human leukocytes and proved to be far more successful and significantly more specific (Mohanty *et al.,* 1997) These methods may have been a more sensitive method but auto fluorescent in plant cells and their extracts may compromise their applicability. Hence why the assay of choice was colorimetric.

The measurement of ROS is complicated, because they are often unstable and highly reactive. Hydroxyl radicals are formed and react with biomolecules very quickly in most biological systems. One method of detection involves the use of a compound that reacts with hydroxyl radicals to form oxidative markers that can be quantitatively measured. The method chosen for this study was the use of a compound known as DMSO to trap hydroxyl radicals and produce methane, which was then quantitatively measured, however this assay did not detect any hydroxyl radical activity. An alternative method uses 8-hydroxy-2-deoxyguanosine which is an excellent marker of

DNA damage in cells and is widely used to determine hydroxyl radical activity (Umegaki *et al.*, 2001). A third method involves the detection of the ascorbyl radical produced from reaction of the hydroxyl radical with ascorbate (Isoda *et al.*, 1995). The drawbacks of these methods are that other ROS, in particular singlet oxygen, have also been shown to cause the formation of 8-hydroxy-2-deoxyguanosine (Wei *et al.,* 1997), therefore it may not give an accurate measurement of hydroxyl radical activity. Also, there are questions surrounding whether or not the actual sample preparation itself may cause additional hydroxyl radical formation.

Spin trapping short lived radical species with nitrone spin traps was, for a period of time, the most reliable method for detecting short lived radical species, however due to the rapid decomposition of the hydroxyl radical adduct and the interference from superoxide forming adducts this method was not ideal (Pou *et al.*, 1989). A recent advancement in electron paramagnetic resonance (EPR) methods has been achieved by spin trapping the secondary carbon centred radicals produced, rather than the initial radical. Using an appropriate substrate, this produced a method of *in vivo* analysis of hydroxyl radical generation that was much more accurate and reliable than any previous methods (Takeshita *et al.*, 2004).

The hydroxyl radical assay presented in this study had sensitivity problems for this set of cultures which may be expected to have a reduced metabolic status. The only samples that produced a positive reading for methane was cell line N, however, the control for this cell line, containing cell line N medium (PGR supplemented) and DMSO also produced methane. Statistically the levels from the control and actual samples were not significantly different, therefore it was concluded that no methane

was produced as a result of hydroxyl radical activity in the sample, but as a result of a component of the medium containing the plant growth hormones (BAP or 2,4-D). This is not inconsistent with the knowledge that certain media components particularly those containing pro-oxidant regulators can produce ROS (Benson, 1990 and 2000). The absence of any hydroxyl radical activity contradicts the explanation for the lack of hydrogen peroxide detected, however if there are very low hydrogen peroxide levels then this would explain the apparent lack of the hydroxyl radical. The results are inconclusive and there is definite scope for the use or development of more sensitive assays (e.g. EPR) for the detection of both hydrogen peroxide and hydroxyl radical activity in these *B. vulgaris* cell lines (see Bailey *et al.*, 1994).

3.7.2.4 Secondary oxidative stress and lipid peroxidation

Summary of lipid peroxidation profiles in habituated *B. vulgaris* cell lines

Peroxidation Product	**Normal (N)**	**Habituated organogenic (HO)**	**Fully habituated (N1)**	**Fully habituated (N3)**
Conjugated dienes Abs at 240nm	4.02	4.68	$10.19^{\uparrow a}$	5.74
Lipid hydroperoxides Abs at 270nm	3.04	2.66	$5.46^{\uparrow b}$	$4.48^{\uparrow b}$
TBARS TBARS index	0.767	0.735	$1.4^{\uparrow a}$	$1.19^{\uparrow a}$
Schiff's bases Organic Abs at 430nm	641.744	526.622	$2154.844^{\uparrow a}$	$1911.17^{\uparrow a}$
Schiff's bases Inorganic Abs at 430nm	58.95	62.81	$167.855^{\uparrow a}$	$97.93^{\uparrow a}$

Table 3.9: Absolute values of lipid peroxidation products in the B. vulgaris cell lines. Statistical differences between the cell lines are marked as follows: ↑a or ↓a = very highly significant, ↑b or ↓b = significant, c = no significant differences, the direction of the arrow denotes whether it is a higher or lower value.

The next stage of the sequence of events in the ROS scenario is lipid peroxidation and the production of secondary oxidative products that arise when ROS attack biomembranes. These were measured to compare the extent of peroxidation occurring in the four *B. vulgaris* cell lines. Data from antioxidant profiles suggest that many of the enzymes involved in the detoxification systems are compromised, particularly in the two fully habituated cell lines (N1 and N3), therefore the extent of the impacts of peroxidation is expected to be greater in these cell lines. Conjugated dienes are the first products formed during lipid peroxidation and levels were very significantly higher in the fully habituated line (N1), suggesting that the extent of lipid peroxidation was higher; the other fully habituated cell line (N3) did not significantly differ, which was unexpected as the activity of several enzymes were reduced to the same extent as the other fully habituated cell line (N1).

Lipid hydroperoxides are the next product formed after conjugated dienes and the assay applied for their measurement showed that both the fully habituated cell lines (N1 and N3) had significantly higher levels than the other two cell lines (HO and N). From the results discussed so far, the fully habituated cell lines (N1 and N3) appear to have a higher level of primary lipid peroxidation products. Lipid hydroperoxides are capable of initiating further lipid peroxidation; therefore a combination of increased ROS and a decrease in antioxidant protection in the fully habituated cell lines will promote further lipid peroxidation, constantly pushing the antioxidant / pro-oxidant ratio further towards the pro-oxidant side, unless the antioxidants can regain control.

Secondary aldehydic products of lipid peroxidation including toxic aldehydes MDA and 4-HNE were quantified using the LPO-586 assay, which is capable of measuring

specifically malondialdehyde and any 4-hydroxyalkenals present. The TBARS assay was also applied which is a more general non-specific assay that gives an overall index of the extent of lipid peroxidation. This measures the level of TBA reactive substances which primarily includes the secondary aldehydes MDA and 4-HNE and other possibly aldehydes associated with sugars. The LPO-586 assay presented many problems when applied to plant tissues investigated in this study which made the interpretation of the results impossible. The assay comprises of two separate determinations - the first assay measures "total MDA + 4-hydroxyalkenals" and the second measures "MDA only". The readings from the "total" assay were lower than the "MDA" assay, which meant that when the MDA values were subtracted from the total values, it produced negative values for levels of 4-hydroxyalkenals. During the assay the samples turned a dark orange colour, which is unexpected as it should be a light pink or purple colour. UV scans were recorded immediately after the assay to determine the maximum absorbance of the samples (see Appendix 6.2) and the orange solution formed appeared to have a maximum absorbance at 469nm and was present in all samples (except the standards), (see Appendix 6.2). This orange colour was not the chromophore that was expected, therefore due to its interference it was concluded that this assay was not suitable for these plant tissue samples.

To date, the LPO-586 assay has been mainly been used in mammalian systems and in some plant embryos (E E Benson, Personal Communications) which may not comprise or contain the same interfering biochemical components found in plant tissue culture media such as sugars. The assay is not widely reported in plant systems, therefore the full extent of assay chromophore and plant/culture medium interference is not known. Due to the age and state of these cell lines the full spectrum of lipid

peroxidation products is not fully identified and the appearance of the orange coloured compound would suggest that other aldehydes or other plant cell components might possibly be interfering with the specificity of the assay. The assay is not therefore recommended for application in *in vitro* plant cultures and if it is used robust studies should be undertaken to determine if interference is present. Therefore these cell lines would benefit from a much more comprehensive review of all lipid peroxidation products using more discerning investigative methods. This would also aid the development of new assays, specifically for use in plant systems.

TBA reactive substances include any aldehydic compounds, such as malondialdehyde, 4-hydroxyalkenals and several other products of lipid peroxidation. This assay has been heavily criticised over the years as it is thought that in plants, pigments and certain sugars may also be reactive towards TBA, risking an overestimation of the results (Cherif *et al.*, 1996 and Hagège *et al.*, 1990). However the TBARS assay is still currently used widely as a general index of lipid peroxidation. The results showed that TBA reactive substances were significantly higher in the two fully habituated cell lines; however as with the LPO-586 assay all samples produced an orange coloured complex, which appeared to be darker in the fully habituated cell lines (N1 and N3). The maximum absorbance of this complex was around 469nm (same as the LPO-586 assay). Red, orange and yellow complexes formed during the TBARS assay have been previously reported to be a result of other lipid peroxidation products (dienals, 2,4-dienals and saturated aldehydes) reacting with TBA (Kosugi and Kikugawa, 1989, Hagège *et al.*, 1990), however they have been reported not to interfere with the TBARS reading at 532nm. The TBARS results backed up the other lipid peroxidation assays where lipid peroxidation products were significantly higher

in the fully habituated cell lines (N1 and N3). There were no significant differences between the normal (N) and habituated organogenic (HO) cell line.

Schiff's bases often referred to as "age pigments" (Lunec and Dormandy, 1979) and are often used as a method of measuring the extent of lipid peroxidation. They are indicators of long-term lipid peroxidation and, generally, increase with age. The data indicates that although all these cell lines were the same age, the levels of both organic and inorganic phases Schiff's bases were significantly higher in the two fully habituated cell lines (N1 and N3) suggesting that cell ageing is not the only factor influencing the formation of Schiff's bases. Increased lipid peroxidation will also result in the formation of Schiff's bases as lipid peroxidation gives rise to more ROS, which can form cross-links with amino side groups of proteins. The data suggests that the two habituated cell lines (N1 and N3) are undergoing increased lipid peroxidation, presumably as a result of increased ROS, due to increased oxidative stress.

This set of lipid peroxidation assays was designed to measure markers of lipid peroxidation, secondary oxidation and long term oxidative stress markers. In the case of the lipid peroxidation products, conjugated dienes and lipid hydroperoxides, which are the primary products of lipid peroxidation, the levels were much greater in the fully habituated cell lines (N1 and N3). Increases in primary products suggests that there is increased oxidative stress occurring in cell lines (N1 and N3), which is as a result of a disturbed antioxidant / pro-oxidant ratio and the severity of the oxidative stress occurring in these cell lines may also suggest that the oxidative stress is prolonged and that there is a significant decrease in antioxidant efficiency. The

secondary products such as MDA (measured as TBARS equivalents) were also significantly higher showing that the primary lipid peroxidation products are not being removed efficiently in these cell lines again suggesting a decrease in enzyme activity. The formation of secondary products has much more serious implications on the cell as the products are often mutagenic (MDA) or highly cytotoxic (4-HNE).

The formation of Schiff's bases (cross-links formed between DNA by ROS) are an indicator of long-term oxidative damage and their accumulation is often observed in aged cell lines. In the fully habituated cell lines the levels of Schiff's bases are so significantly higher that it suggests that these cell lines are, and have been, under significantly higher oxidative stress for some time and this combined with decreased antioxidant protection will have serious implications for membrane stability and cell viability.

3.7.3 DNA Methylation: examining a potential role in habituation and neoplasia

This study was considered of importance because DNA methylation is associated with epigenetic changes, which could be involved in neoplastic progression. The first consideration was to develop a suitable extraction and analysis method for DNA for cultures that are potentially difficult to characterise at the molecular level due to their unusual physiologies. The CTAB method was therefore chose as it had been applied previously to aged cultures (Harding *et al.*, 1996). This uses high salt extraction, which entails five main stages. Cell lysis involves disruption of the cells and degradation of the proteins and RNA, the extraction step includes a chloroform extraction, which causes protein denaturation and separation of the aqueous and

organic layers. The precipitation steps use a CTAB buffer, which frees the DNA from the buffer and precipitates it out of solution. Purification steps follow where the pellet of DNA is washed with ethanol and finally the pellet is re-suspended in TE buffer which preserves the DNA till required for analysis. This particular method was chosen as it has been used successfully in many plant systems as it eliminates polysaccharides from the samples, which are often in high concentrations in plant systems and can cause sample interference. This assay is relatively economical to carry out, however it is labour intensive.

DNA content was measured in the four *B. vulgaris* cell lines and the extracted DNA was then used to carry out the HPLC based assay to determine the extent of DNA methylation. The method chosen for this assay was based on a HPLC derived method described by Matassi, *et al.* (1992) and is designed to quantify the levels of cytosine and 5-methylcytosine in the sample. A HPLC based method was chosen as it has been successfully applied in plant systems previously (Demeulemeester, *et al.*, 1999; Fojtová, *et al.*, 2001 and Matassi, *et al.*, 1992) and is a very sensitive, reliable and globally used method of analysis.

The quantity of DNA extracted, particularly in the two fully habituated cell lines, was extremely low and this reflected on the HPLC assay results. The lower recovery of DNA may be attributed in part, if not substantially to the fact that these cultures are dedifferentiated aged and largely non-meristematic. In the case of the organogenic cultures, their abnormal hyperhydric features would predispose them to a lower (on the basis of fresh weight) yield of DNA relative to plant tissues that are competent and juvenile. Although DNA methylation was only detected in the two fully habituated

cell lines (N1 and N3), the levels were very low and highly variable, rendering the results insignificant. It was therefore concluded that the CTAB extraction method, based on 5g fresh weight material, provided insufficient DNA for this assay in these cultures. There are unresolved issues with DNA extractions and in this case the main problem using the CTAB method was the inability of the DNA to precipitate from the solution and any DNA that did eventually manifest turned black in colour. A phenomenon which has been observed in other plant species by Harding (2004) who compared the most commonly used DNA extraction procedures on *Vitis vinifera* in glasshouse plants, *in vitro* plantlets and callus cultures. The CTAB method extracted the highest yield of DNA in both glasshouse plants and *in vitro* plantlets but in callus cultures the CTAB method yielded slightly lower DNA than a chloroform / phenol extraction procedure (Harding, 2004). The phenol / chloroform extraction procedure is a commonly used method, where phenol is employed to disassociate the proteins from the DNA and the chloroform denatures the proteins and lipids and helps maintain a good clean organic / aqueous separation. The disadvantages of using this method are that phenol must first be buffered and be completely free from any oxidative substances and both chloroform and phenol are toxic and care should be taken when handling. However, this method might be better for the extraction of DNA from the *B. vulgaris* cell lines.

Other alternatives include a silica resin based method, where after homogenisation and lysis the cells are passed through a silica resin column, where the RNA passes through the column in solution and the DNA binds strongly to the silica matrix. DNA can then be isolated by eluting with another solution that frees the DNA, whereupon it can be precipitated and stored in TE buffer. This method is fast and safer to perform

than the phenol / chloroform procedure, it is also very efficient and has been shown to have a 80% extraction success rate (Tian *et al.,* 2000) and might prove a useful alternative method for DNA extractions in plants in future studies.

3.8 CONCLUSIONS

The aim of this study was to determine the morphological and biochemical characteristics of four *B. vulgaris* cell lines that are at various stages of habituation, to determine whether oxidative stress and the resulting ROS were involved in the habituation process. A summary of the main findings from this current study is detailed below:

- The morphological studies conducted in the normal cell line (N) demonstrated that there were cytological abnormalities, which were previously only observed in the fully habituated cell lines. This would suggest that since the last comprehensive study carried out these cultures some changes have occurred, similar to that of the cancerous (fully habituated) cell lines N1 and N3.
- The activities of several enzymatic antioxidants in the normal cell line (N) were decreased, however not to the same extent as the two fully habituated cell lines (N1 and N3). However, unlike the two fully habituated cell lines, the normal cell line still maintained relatively high levels of other non-enzymatic antioxidants which appeared to be able to compensate to a certain degree for the loss of enzymatic antioxidants.

- The glutathione ratio in N was only slightly reduced (20% decrease in reduced glutathione levels) compared to what would be expected in normal healthy cells.
- This (N) cell line also had the lowest levels of lipid peroxidation products, suggesting that the antioxidants were still relatively proficient in the removal of ROS, and hence the preventing the launch of cascading free radical reactions, which lead to lipid peroxidation.
- Therefore it appears that the normal cell line, although under some degree of oxidative stress, is still capable of just maintaining an effective antioxidant / pro-oxidant equilibrium.
- The habituated organogenic cell line (HO) had several morphological abnormalities; however since the last comprehensive study there did not appear to be many additional morphological changes.
- The biochemical characteristics of this (HO) cell line are not significantly different from the normal cell line, however generally their antioxidant levels were slightly lower than the normal cell line, but still significantly higher in most cases than the fully habituated cell lines.
- Levels of lipid peroxidation products in this (HO) cell line were significantly lower than the fully habituated cell lines, suggesting again as is the case with cell line N, other antioxidants such as glutathione and possibly ascorbate were able to compensate for the lack of other enzymes.
- The fully habituated cell lines (N1 and N3) have significant morphological, cytological and biochemical abnormalities.

- The antioxidant profiles of these fully habituated cell lines demonstrated a complete depletion in antioxidant capacity and this is most likely due to the inability to remove ROS and exacerbated by secondary oxidative stress.
- Primary antioxidant enzymes (SOD, peroxidase, and catalase) were low in N1 and N3 and glutathione components which were relatively high in the other cell lines were almost completely depleted, which was associated with significantly increased lipid peroxidation products.
- The overall profile of the fully habituated cell lines, are that antioxidant protection has been compromised to a critical point, and that levels of pro-oxidants have increased to a level where the equilibrium between the two has been completely disrupted.
- The fully habituated cells appear incapable of regaining redox control and hence morphogenetic competence is lost and, as a consequence, they have reached an irrevocable point in their neoplastic progression.

4.0 CHAPTER FOUR: A STUDY OF *IN VITRO* AGEING IN *G. MAX*

4.1 INTRODUCTION

Two *G. max* cell lines denoted SW and SG have been maintained in *in vitro* tissue culture for approximately sixteen years and can therefore be considered as "aged" cultures. Both cell lines are dedifferentiated callus and are non-embryogenic. These will be used to explore by profiling antioxidant potential and levels of ROS and lipid peroxidation products, the role of oxidative stress in long-term *in vitro* ageing.

4.2 EXPERIMENTAL AIMS AND OBJECTIVES OF *G. MAX* STUDY

1. To study the morphological characteristics of both *G. max* cell lines

2. To profile the activity of antioxidants in the two *G. max* cell lines

3. To measure the levels of reactive oxygen species (ROS) and lipid peroxidation products of both *G. max* cell lines

RESULTS AND DISCUSSION

4.3 CULTURE GROWTH AND VIABILITY MEASUREMENTS

The following section presents a morphological overview of the two *G. max* lines, SW and SG. Growth characteristics and structural differences will be identified, using growth experiments, microscopic analysis and staining techniques.

4.3.1 Growth analysis

Sample ID	**Percentage gain fresh weight (g) over a thirty day period**
G. max white (SW)	1033%
G. max green (SG)	713%

Table 4.1: Increase in fresh weight over a thirty-day subculture cycle for *G. max* cell lines SW (white) and SG (green).

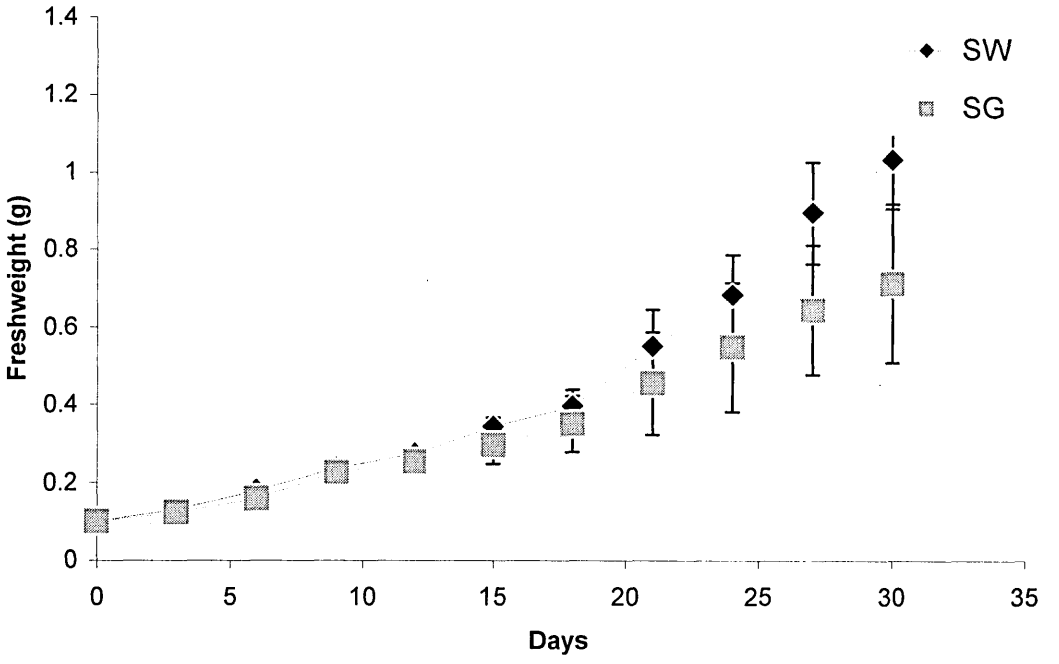
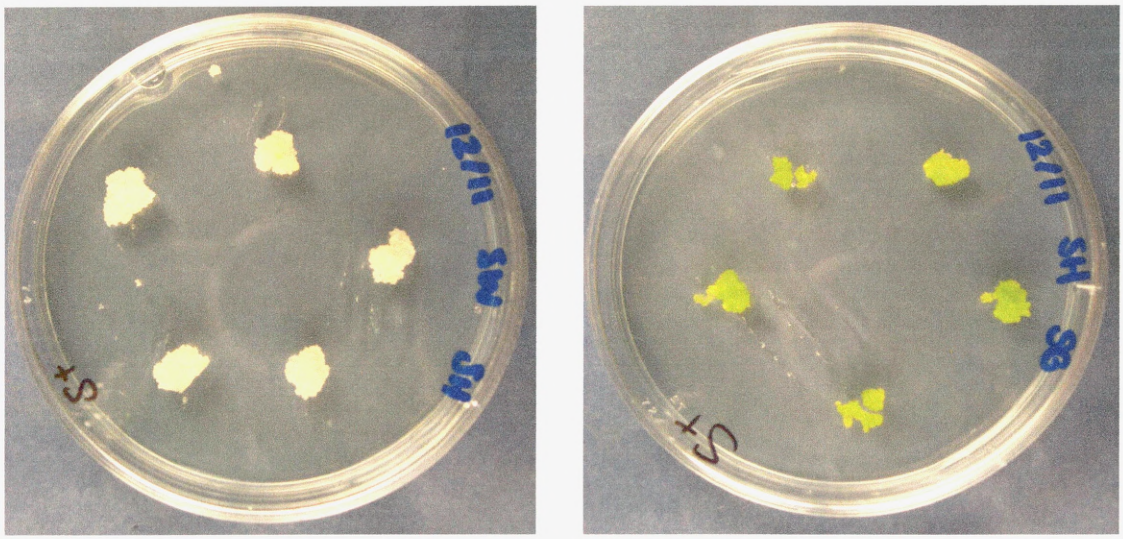


Figure 4.1: Growth curve for *G. max* cell lines SW (non-pigmented) and SG (pigmented). Data points were calculated as the means of replicate samples, where n=9. The data was collected every 3 days and the results are expressed as a fresh weight value. Errors are derived from the standard deviations of the data series.

Data show that cell line *G. max* (SW) gained more fresh weight over the thirty-day period; however the growth patterns for the two cell lines were not significantly different (see Table 4.1). The exponential growth phase commenced at around day five and after that increased steadily and both cell lines showed a decline in growth around day thirty.

4.3.2 General structural analysis



Scale: 9cm (Relative to Petri dish size)

A **B**

Figure 4.2: Digital images of aged *G. max* cell lines SW and SG. Image A: Dedifferentiated callus (SW) maintained on medium supplemented with PGRs (NAA and 2,4-D) - this callus appears compact, however is deficient in chlorophyll. Image B: Dedifferentiated callus (SG), maintained on medium supplemented with PGRs (NAA and 2,4-D) - this callus appears normal and compact and contains chlorophyll. Scale is presented as relative to a 9 cm Petri dish.

4.3.3 *Microscope analysis (X 100 magnification)*

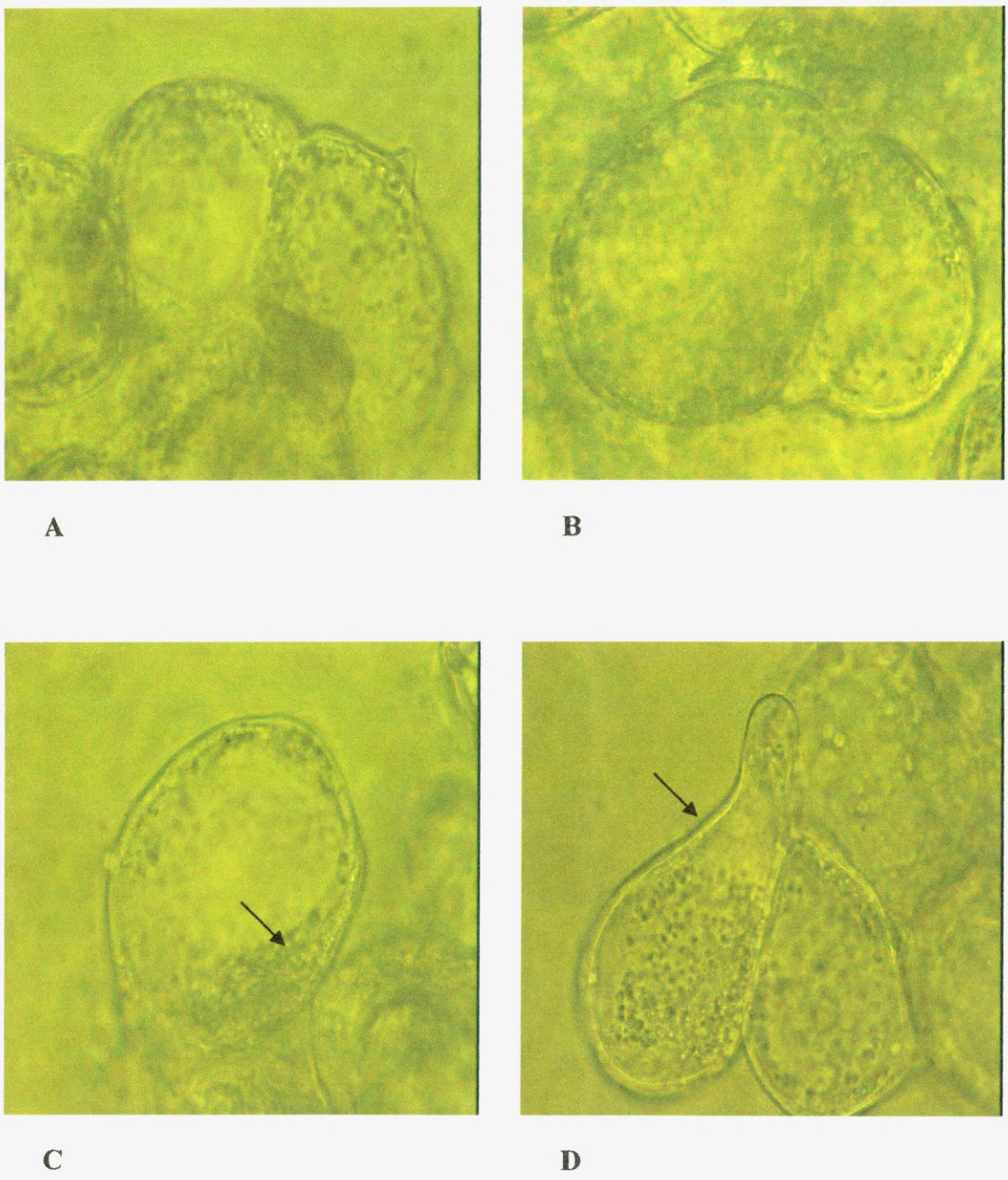


Figure 4.3: Digital images from a light microscope (X100). Images A and B: *G. max* (SW), cell elongation observed and granular material accumulated around the inside of the membranes with large vacuoles. Images C and D: *G. max* (SG), cell elongation observed and large vacuoles are seen with granular material appearing around the edges of the membranes.

4.3.4 Viability testing (100X magnification) with fluoroscein diacetate (FDA)

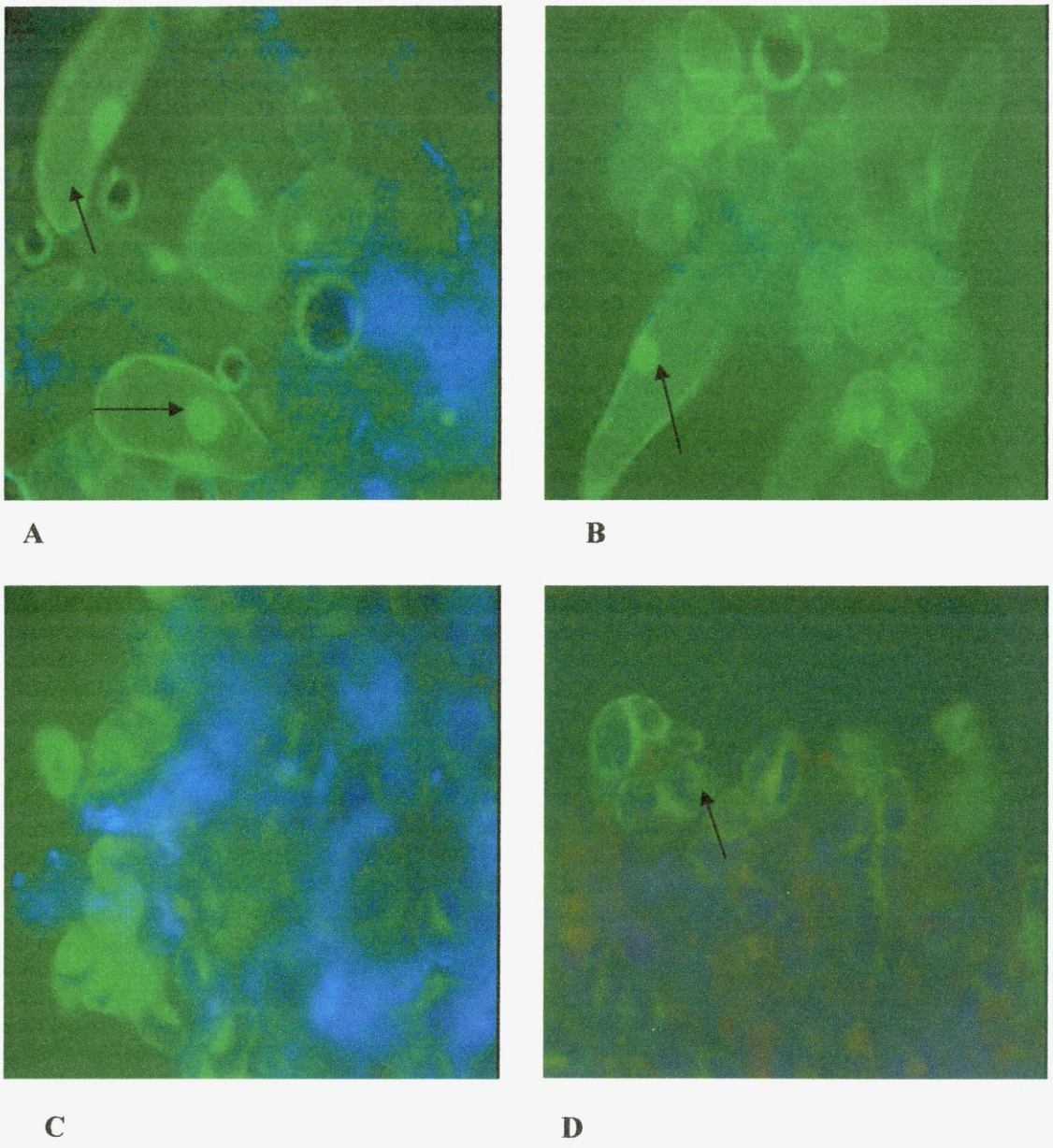


Figure 4.4: Digital images of *G. max* captured under a microscope, with a fluorescein diacetate (FDA) stain applied (blue colouration are non-viable cells, green colouration indicates viable cells). Image A and B, taken from *G. max* (SW) shows cells that are elongated with clear smooth membranes and highly fluorescent spots, which represent areas of elevated esterase activity, such as the nucleus. Images C and D: taken from the *G. max* (SG). The cells appear smaller, are more spherical in shape and have fluorescence areas throughout, making it more difficult to identify the nucleus; however in some cells the nucleus area is visible. Note stain application was used primarily to elucidate the morphological status rather than viability.

G. max Cell line	Pigment	Organised structures	Abnormalities	Hyperhydric behaviour	FDA viability
Soya White (SW)	Yellow colouration darkened throughout subculture period, no chlorophyll pigentation	None (dedifferentiated)	Cell elongation visible, however there was granular material that appeared to accumulate around the edges of the membrane	No	Small areas appeared to fluoresce brightly indicating high esterase activity (cell growth/ division)
Soya Green (SG)	Chlorophyll pigment, dark green colouration maintained throughout subculture period	None (dedifferentiated)	Cells were more spherical, some elongation visible and granular material accumulated around the membranes	No	Small areas appeared to fluoresce brightly indicating high esterase activity (cell growth/ division)

Table 4.2: Summary of morphological findings from *in vitro* aged *G. max* cell lines

4.4 BIOCHEMICAL ANALYSIS OF *IN VITRO* AGED *G. MAX*

Biochemical analyses were carried out using similar assays as used in the preceding Chapter 3 and may be categorised again accordingly:

- Antioxidant assays
- Reactive oxygen species (ROS) detection
- Lipid peroxidation products

4.4.1 Antioxidant assays

The activities of the following antioxidant enzymes Cu,Zn-superoxide dismutase (SOD), catalase, peroxidase, glutathione reductase and glutathione S-transferase were determined and activity expressed as the change in absorbance per mg of protein. In addition, the levels of the antioxidants glutathione and ascorbate were calculated (expressed as a concentration per g fresh weight) and the redox status was determined and, finally, the levels of protein and non-protein bound sulphydryls were calculated. Determining all of the above allows an assessment of the overall antioxidant protection status of the *G. max* cell lines to be made.

4.4.1.1 Protein analysis

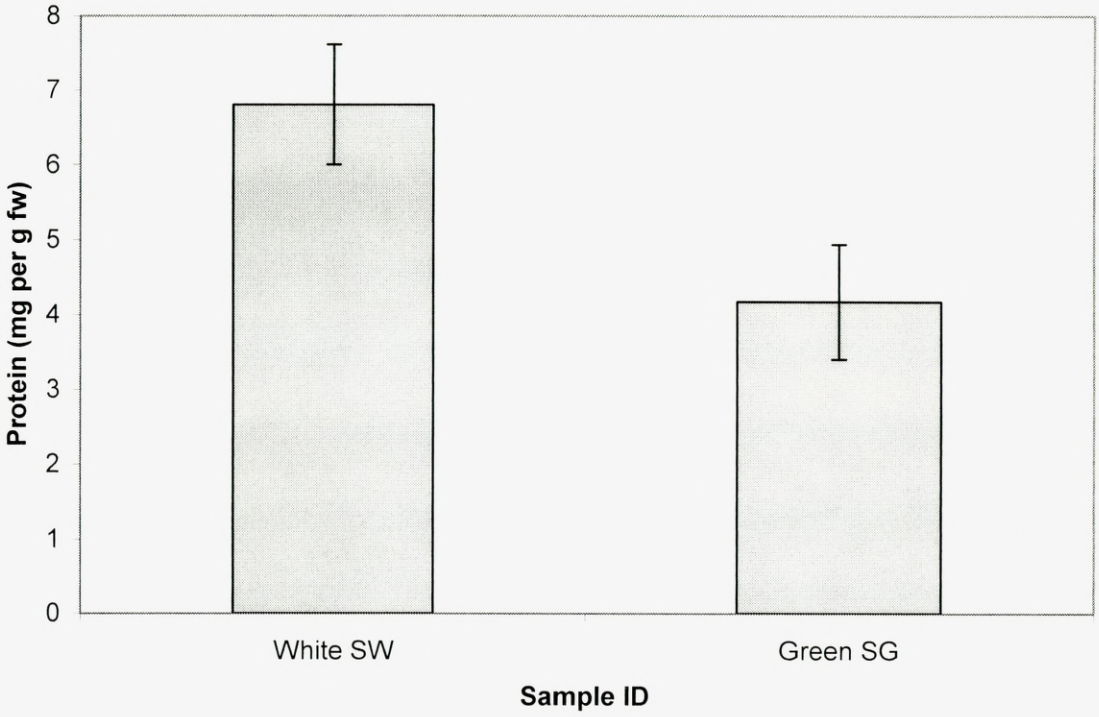


Figure 4.5: Protein concentration in *G. max,* White (SW) and Green (SG). Data points were calculated as the means of replicate samples, where N=9. Concentration is based per g of fresh weight material. Errors are derived from the standard deviations of the data series.

The data showed that there was a highly significant difference between the two *G. max* lines ($P<0.01$, $F=51.49$) with the level of protein being higher in the white cell line (SW). ANOVA assumptions were satisfied for this data series

4.4.1.2 Superoxide dismutase activity

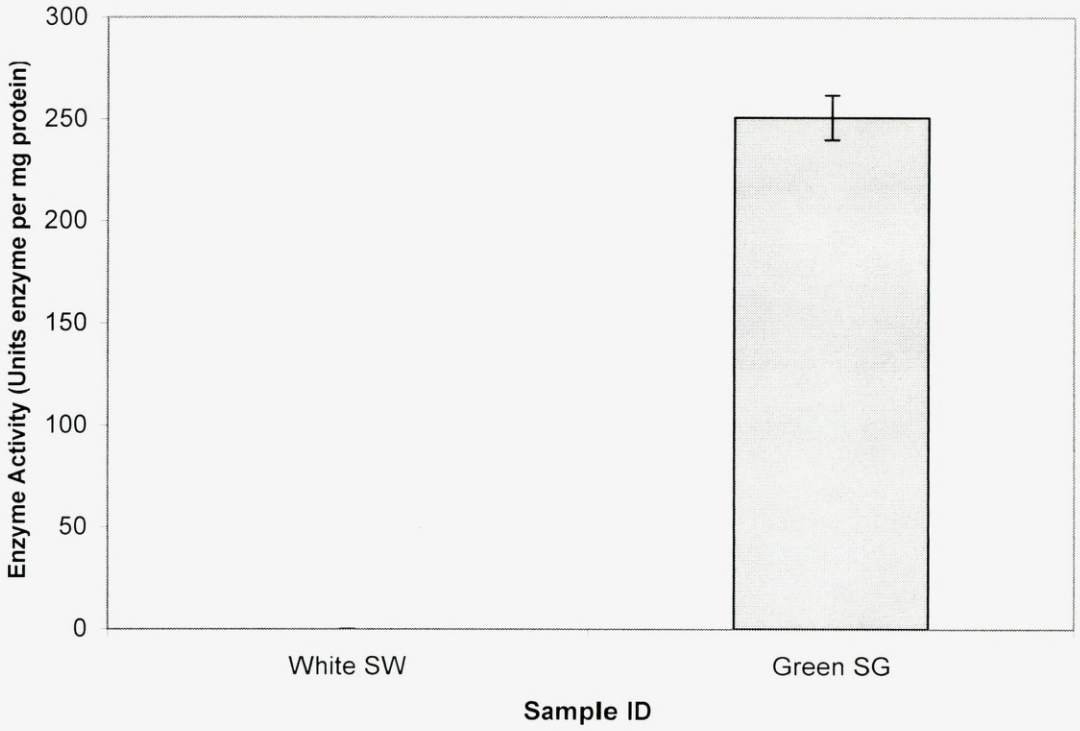


Figure 4.6: Superoxide dismutase activity in *G. max*, White (SW) and Green (SG). Data points were calculated as the means of replicate samples, where N=9. Activity is based on units of enzyme per mg of protein. Errors are derived from the standard deviations of the data series.

Superoxide dismutase was only detected in the green (SG) cell line and therefore the results were highly significant. The data were analysed statistically using ANOVA one-way analysis ($P<0.01$, $F=369.47$) and ANOVA assumptions were satisfied for this data series.

4.4.1.3 Catalase activity

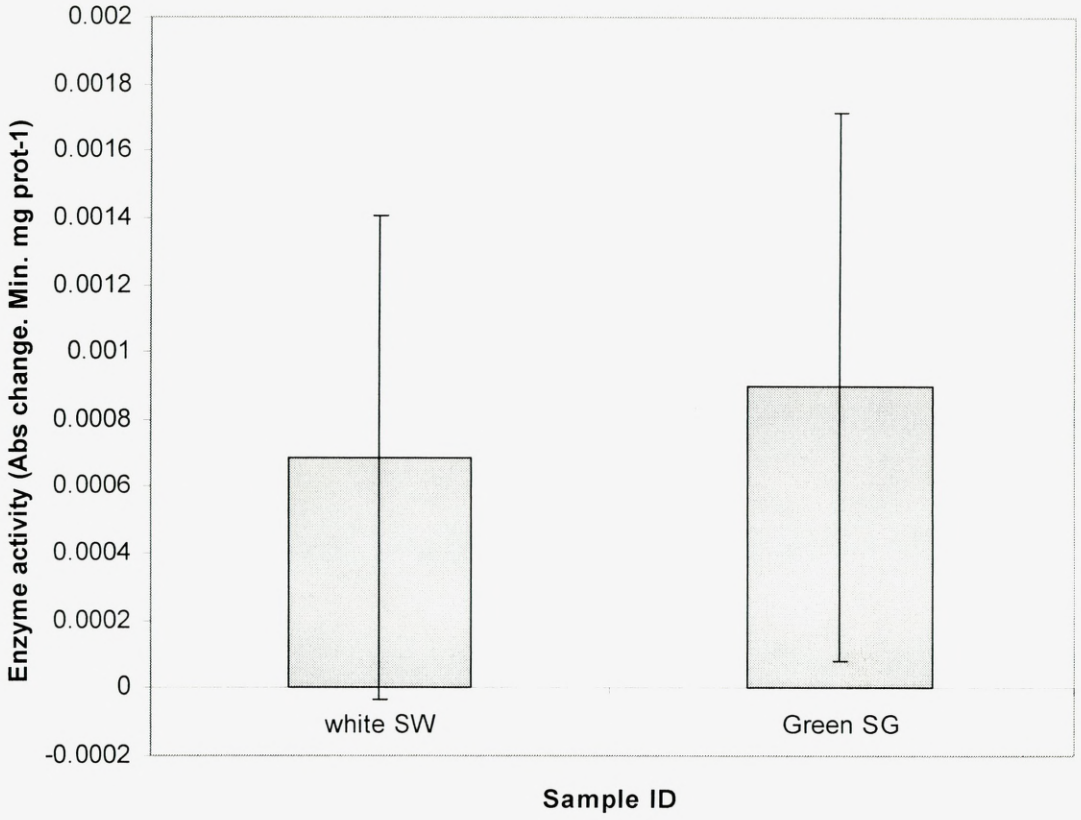


Figure 4.7: Catalase activity in *G. max*, White (SW) and Green (SG). Data points were calculated as the means of replicate samples, where N=9. Enzyme activity is based on the absorbance change per min per mg of protein. Errors are derived from the standard deviations of the data series.

Catalase activity was extremely low in both cell lines (0.0006-0.0009 Abs change. $min.gfw^{-1}$). The data showed that there was no significant difference between the two cell lines ($P>0.05$, $H=0.94$). The Kruskall-Wallis test was applied, as the data series did not have normal distribution.

4.4.1.4 Peroxidase activity

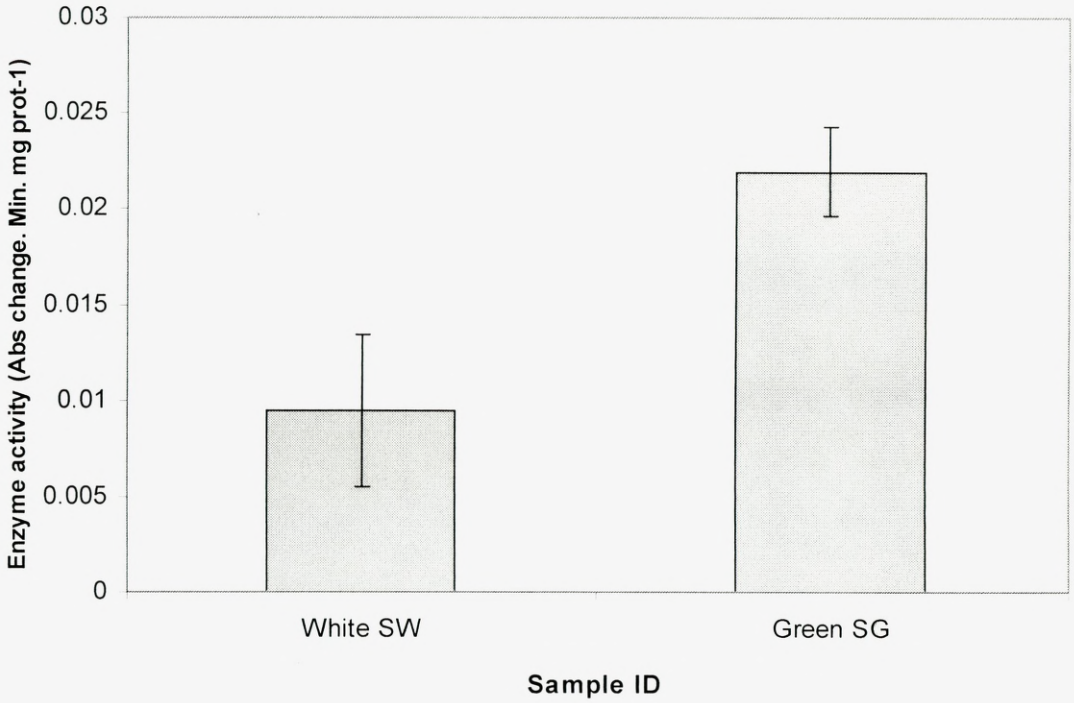


Figure 4.8: Peroxidase activity in *G. max*, White (SW) and Green (SG). Data points were calculated as the means of replicate samples, where N=9. Enzyme activity is based on the absorbance change per min per mg of protein. Errors are derived from the standard deviations of the data series.

There was a significant difference between the two cell lines but on statistical analysis the data series did not satisfy all the ANOVA assumptions, as the data was non-parametric, the Kruskall-Wallis test was performed ($P<0.05$, $H=4.31$). Peroxidase levels were considerably lower in the white *G. max* line (SW).

4.4.1.5 Glutathione

Total glutathione

Figure 4.9: Total glutathione concentration in *G. max*, White (SW) and Green (SG). Data points were calculated as the means of replicate samples, where N=9. Concentration is expressed in mM and is based per gram of fresh weight material. Errors are derived from the standard deviations of the data series.

There was a significant difference between reduced glutathione levels in the two *G. max* cell lines. The data series was non-parametric therefore one way ANOVA analysis could not be used. The Kruskall-Wallis test was selected to analyse the data and it was found that total glutathione was significantly lower in the green cell line (SG), ($P<0.05$, $H=4.49$).

Oxidised glutathione (GSSG)

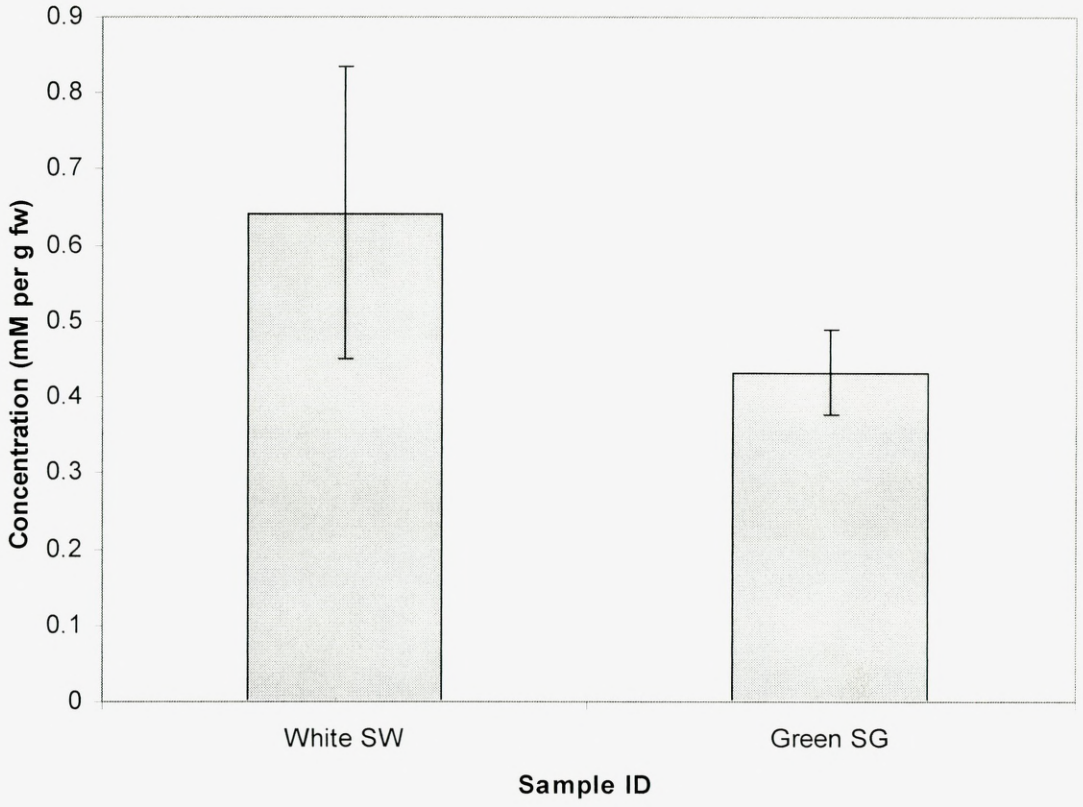


Figure 4.10: Oxidised glutathione (GSSG) concentration in *G. max*, White (SW) and Green (SG). Data points were calculated as the means of replicate samples, where N=9. Concentration is expressed in mM and is based on per gram of fresh weight material. Errors are derived from the standard deviations of the data series.

The oxidised glutathione levels did not significantly differ between the two cell lines ($P>0.05$, $H=0.03$) and, since the data was non parametric, the Kruskall-Wallis test was used to statistically analyse the data series.

Comparison of reduced and oxidised glutathione

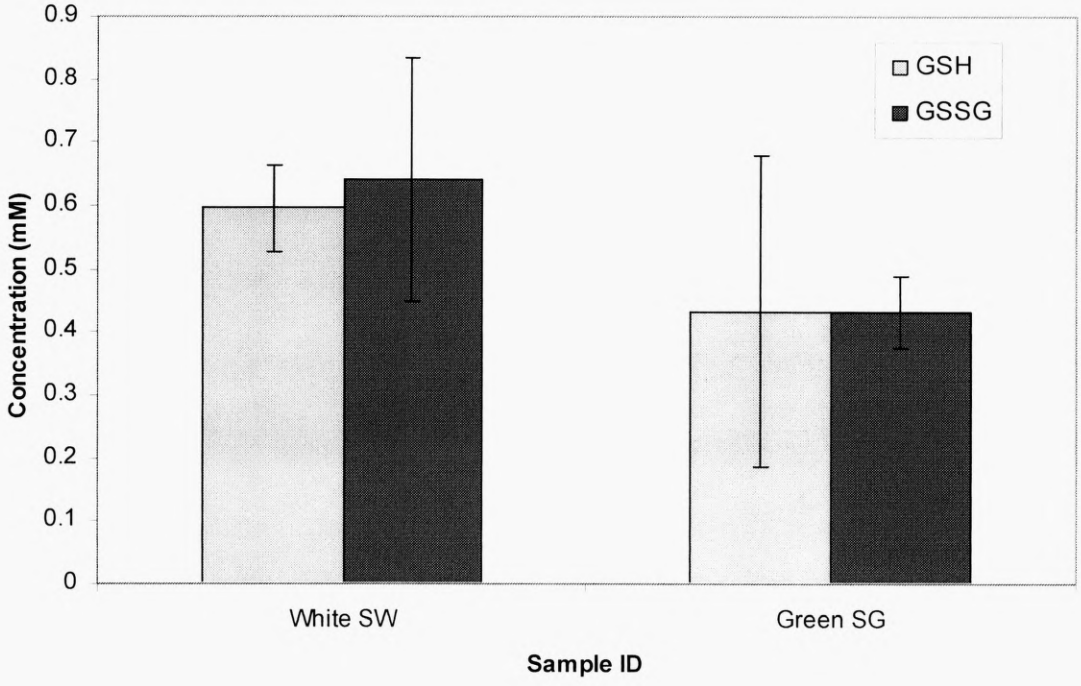


Figure 4.11: Comparison of reduced and oxidised glutathione in *G. max*, White (SW) and Green (SG). Data points were calculated as the means of replicate samples, where N=9. Concentration is expressed in mM and is based on per gram of fresh weight material. Errors are derived from the standard deviations of the data series.

Sample ID	Glutathione redox status
G. max (SW)	0.4812
G. max (SG)	0.5000

Table 4.3: Glutathione redox status in *G. max* cell lines

The glutathione redox status of the two cell lines was not significantly different. In both there was a significant decrease in the reduced glutathione levels, as compared to normal cells which are thought to have a glutathione ratio around 0.9 (Carvalho and Amâncio, 2002).

4.4.1.6 Ascorbate assay

Total ascorbate

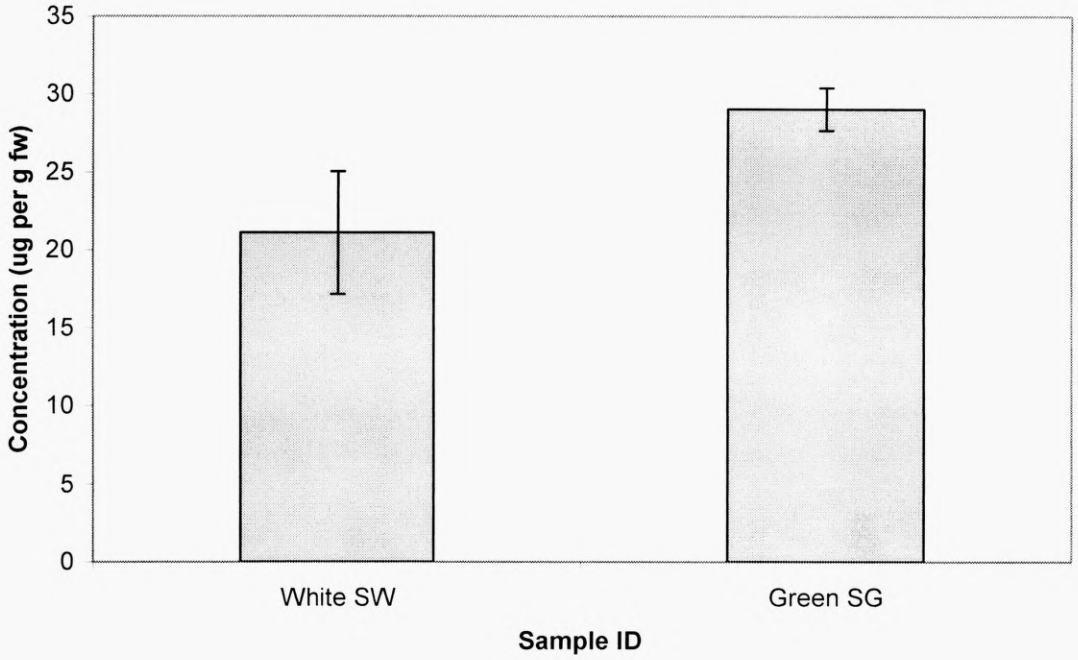


Figure 4.12: Total ascorbate and dehydroascorbate concentration in *G. max*, White (SW) and Green (SG). Data points were calculated as the means of replicate samples, where N=9. Concentration is expressed in micrograms and is based on per gram of fresh weight material. Errors are derived from the standard deviations of the data series.

There was a significant difference in ascorbate levels in the two cell lines ($P<0.05$, $F=13.71$). ANOVA was used to analyse all the data in the ascorbate assays and ANOVA assumptions were satisfied in both cases. Total ascorbate was higher in the pigmented green *G. max* line (SG).

Ascorbate

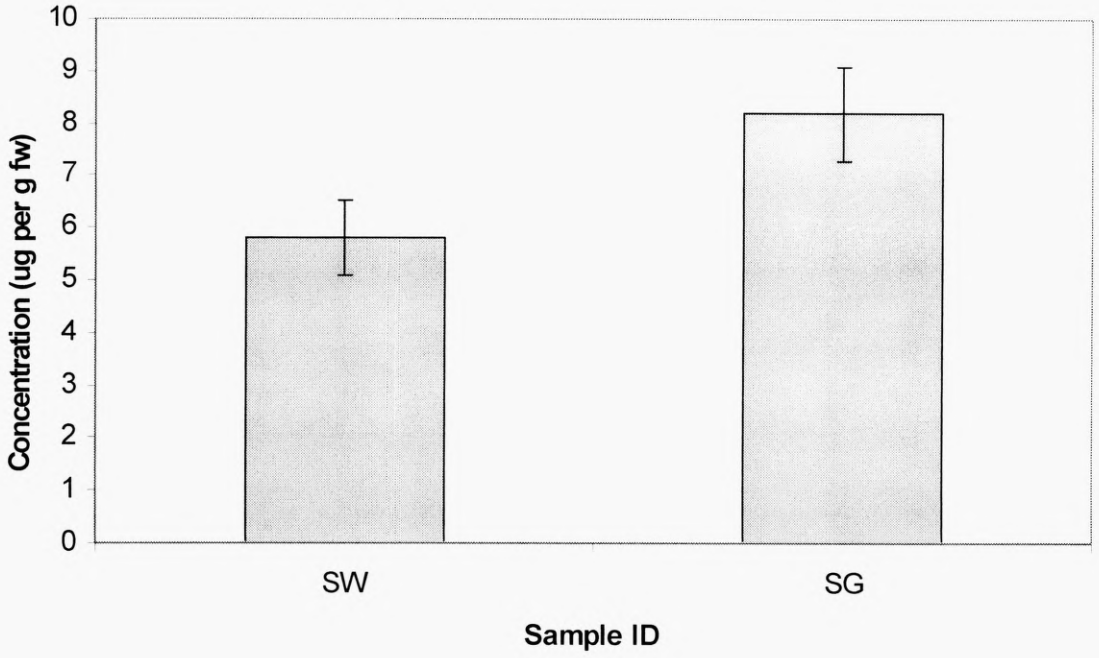


Figure 4.13: Ascorbate concentration in *G. max*, White (SW) and Green (SG). Data points were calculated as the means of replicate samples, where N=9. Concentration is expressed in micrograms and is based on one g of fresh weight material. Errors are derived from the standard deviations of the data series.

The data showed that there was a highly significant difference between the two cell lines ($P<0.01$, $F=40.25$). There were higher levels of ascorbate in the pigmented line (SG). ANOVA assumptions were all satisfied for this data series.

Comparison of reduced and oxidised ascorbate

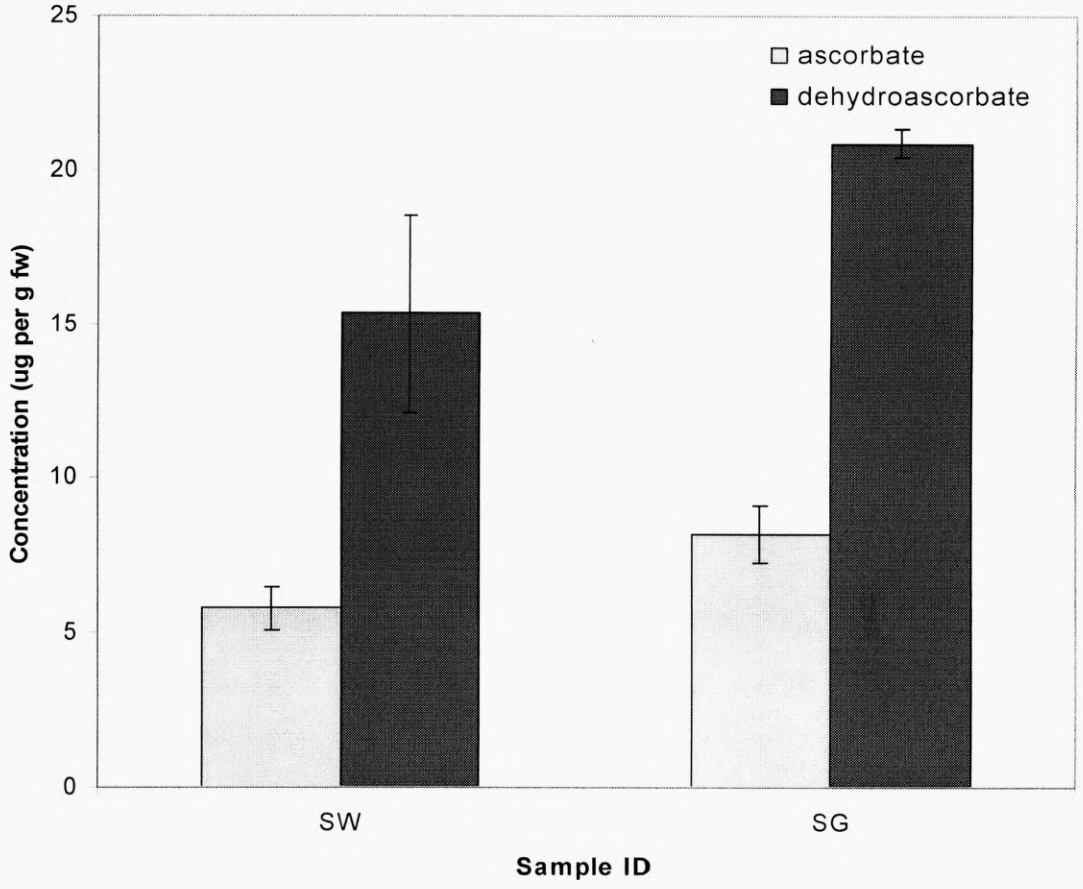


Figure 4.14: Comparison of ascorbate and dehydroascorbate in *G. max*, White (SW) and Green (SG). Data points were calculated as the means of replicate samples, where N=9. Concentration is expressed in micrograms and is based on one gram of fresh weight material. Errors are derived from the standard deviations of the data series.

Cell line	Ascorbate redox status
G. max (SW)	0.38
G. max (SG)	0.28

Table 4.4: Ascorbate redox status in *G. max* cell lines

The comparison of reduced and oxidised ascorbate shows that there is no significant difference in the ascorbate redox status (see Table 4.4) in the two *G. max* cell lines.

4.4.1.7 Sulphydryl groups

Total sulphydryl groups

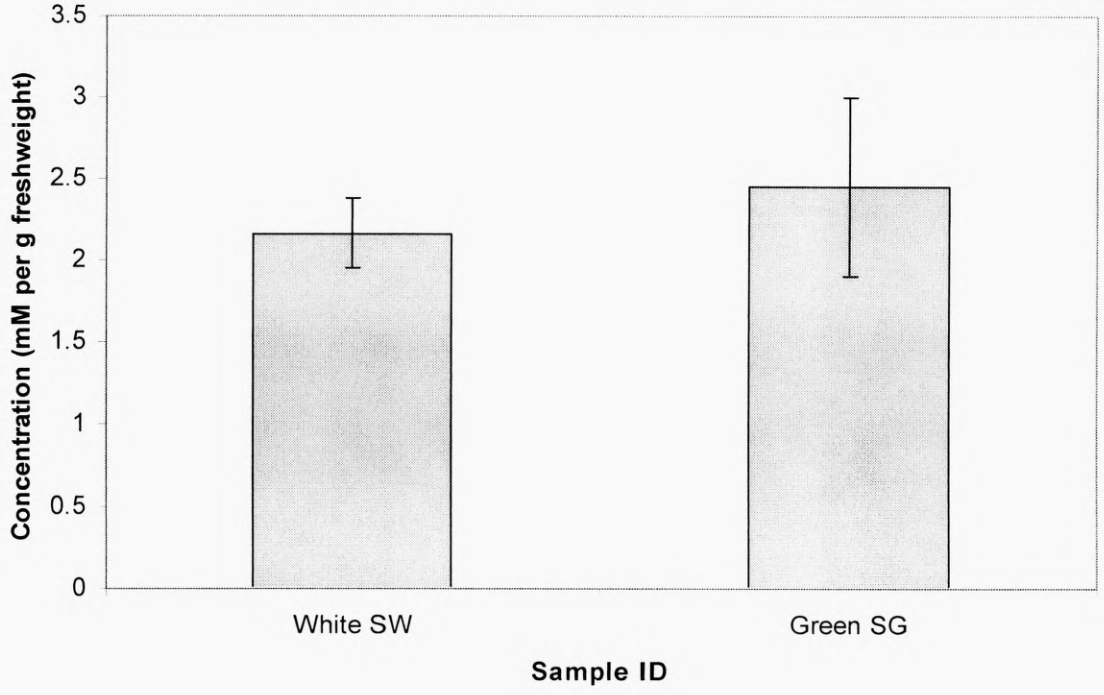


Figure 4.15: Total sulphydryl concentration in *G. max*, White (SW) and Green (SG). Data points were calculated as the means of replicate samples, where N=9. Concentration is expressed in micro moles and is based on one gram of fresh weight material. Errors are derived from the standard deviations of the data series.

The data indicated that there was no significant difference in total SH groups between the two cell lines ($P>0.05$, $F=1.30$). ANOVA one-way analysis was used to analyse the data series and in both assays ANOVA assumptions were satisfied.

Non-protein bound sulphydryl groups

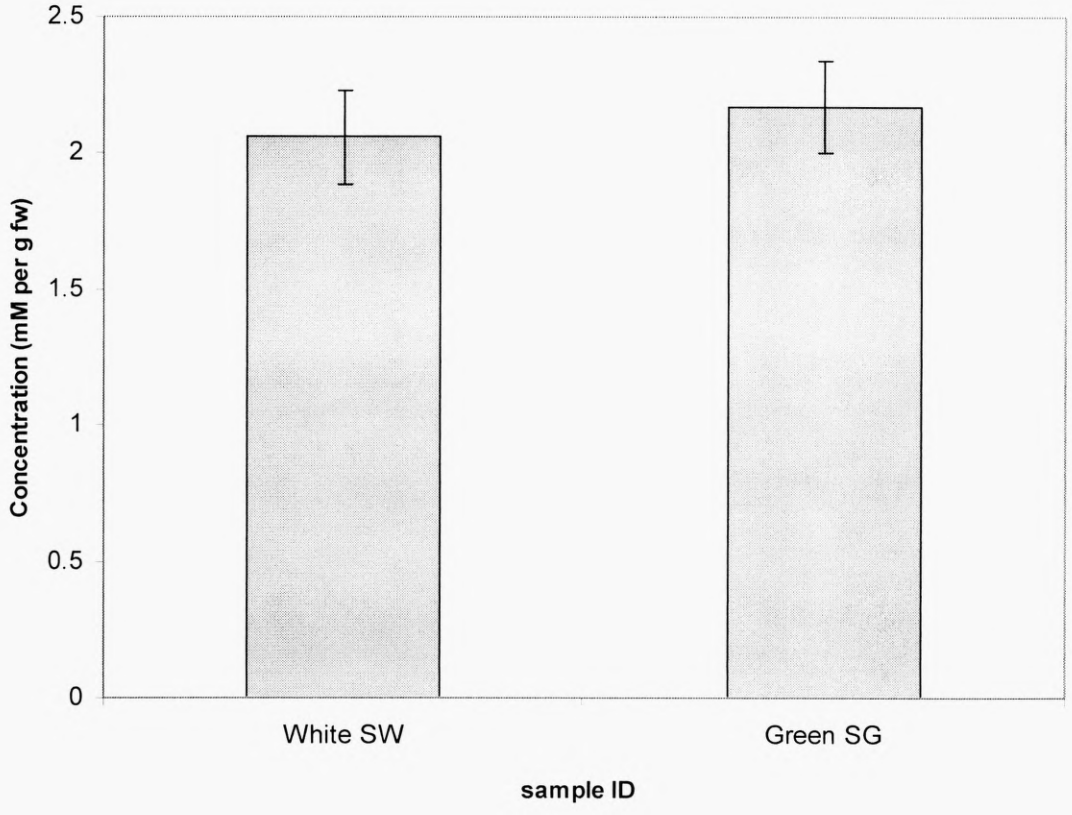


Figure 4.16: Non-protein bound sulphydryl concentration in *G. max*, white (SW) and *G. max,* green (SG). Data points were calculated as the means of replicate samples, where N=9. Concentration is expressed in micrograms and is based on one gram of fresh weight material. Errors are derived from the standard deviations of the data series.

There was no significant difference in non-protein bound sulphydryl groups between the two *G. max* cell lines. ANOVA one-way analysis was used to statistically analyse the data series ($P>0.05$, $F=0.73$), the data satisfied all ANOVA assumptions.

Comparison of protein and non-protein bound sulphydryls

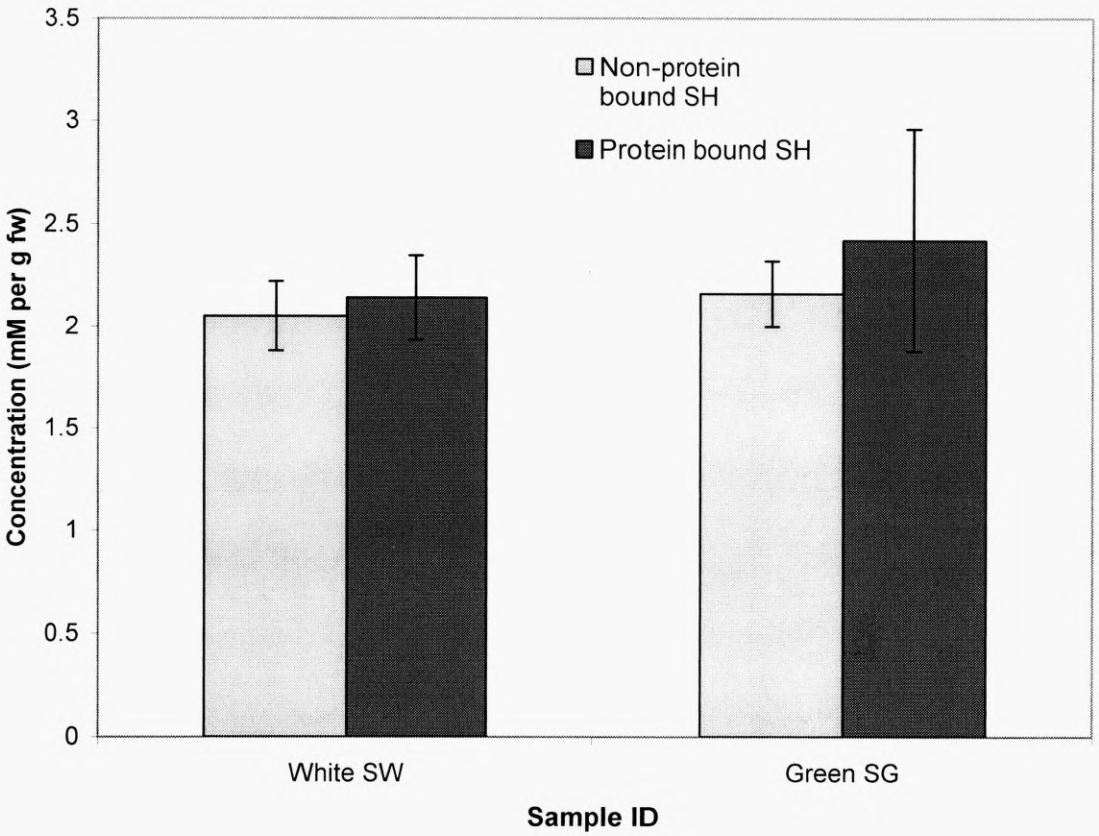


Figure 4.17: Comparison of sulphydryl groups in *G. max*, White (SW) and Green (SG). Data points were calculated as the means of replicate samples, where N=9. Concentration is expressed in micro grams and is based on one gram of fresh weight material. Errors are derived from the standard deviations of the data series.

The protein bound and non-protein bound sulphydryl groups are almost equal in the *G. max* cell lines, suggesting that the protein bound sulphydryl groups are being maintained and not are oxidised extensively through lipid peroxidation, as is the case in the *B. vulgaris* cell lines (N1 and N3).

4.4.1.8 Glutathione reductase activity

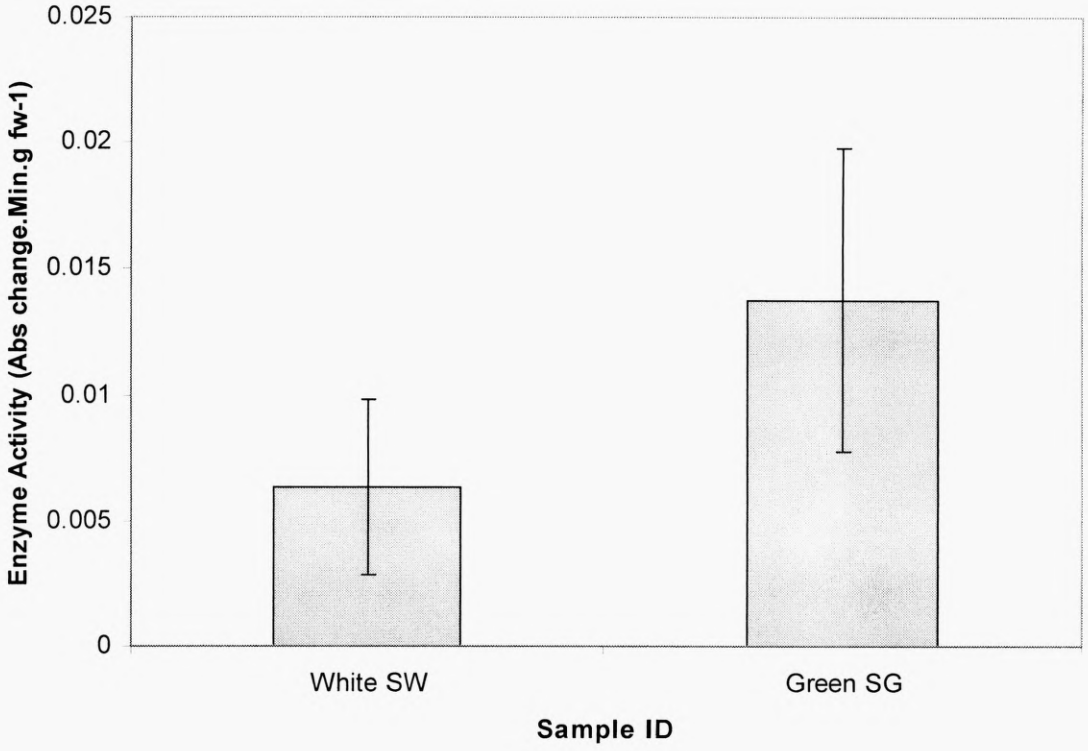


Figure 4.18: Glutathione Reductase activity in *G. max*, White (SW) and Green (SG). Data points were calculated as the means replicate samples, where N=9. Enzyme activity is based on the absorbance change per min per mg of protein. Errors are derived from the standard deviations of the data series.

There was no significant difference in glutathione reductase activity in the two *G. max* cell lines. ANOVA assumptions were satisfied for this data series and the data was analysed using one-way ANOVA ($P>0.05$, $F=0.01$).

4.4.1.9 Glutathione S-transferase activity

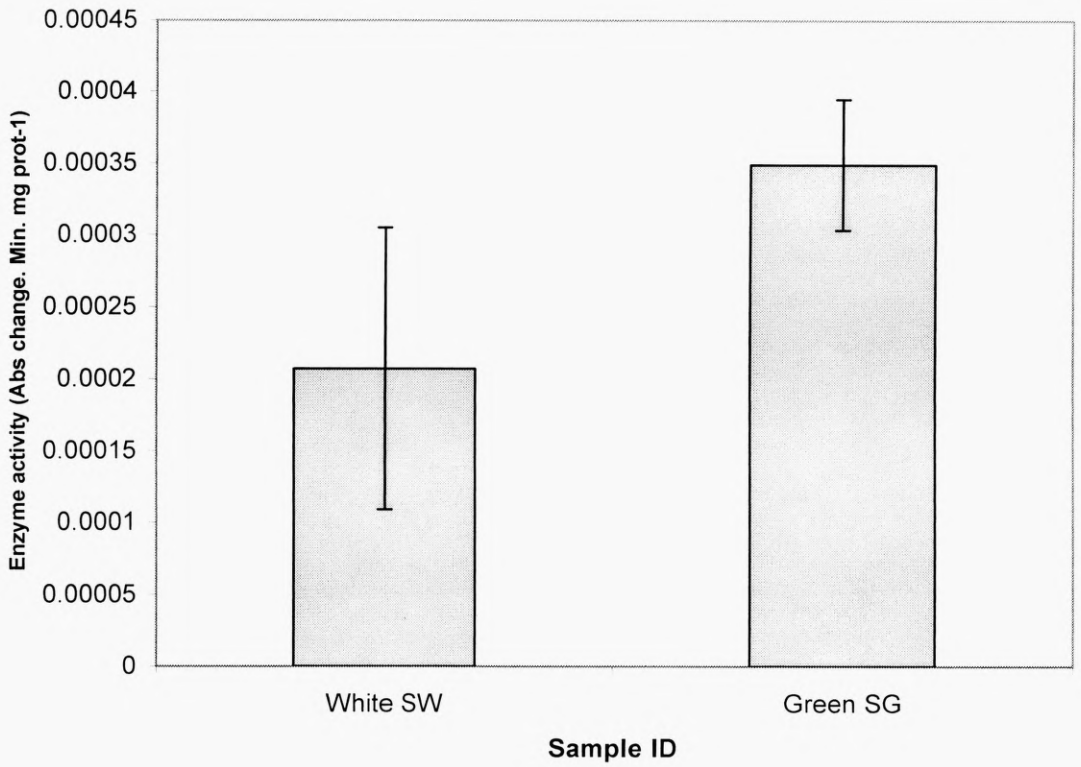


Figure 4.19: Glutathione S-transferase activity in *G. max*, White (SW) and Green (SG). Data points were calculated as the means of replicate samples, where N=9. Enzyme activity is based on the absorbance change per min per mg of protein. Errors are derived from the standard deviations of the data series.

There was no significant difference between the glutathione S-transferase activity in the two *G. max* cell lines ($P>0.05$, $F=0.03$). One-way ANOVA was used to statistically analyse the data and all ANOVA assumptions were satisfied.

4.4.2 Reactive oxygen species (ROS)

4.4.2.1 Hydrogen peroxide (H_2O_2)

No hydrogen peroxide was detected in either of the *G. max* cell lines at the limits of detection, which are 10µL/mL (30% H_2O_2) for this assay.

4.4.2.2 Hydroxyl radical

Hydroxyl radical activity was measured using DMSO as a radical trap to produce methane, which was then measured by gas chromatography. No methane was produced in these *G. max* cell lines, indicating that there was no hydroxyl radical activity detected or that the hydroxyl activity was below the limits of detection which is 15.69ppm per mL headspace gas.

4.4.3 Lipid peroxidation products

The following section presents the data collected during the measurement of the lipid peroxidation products including conjugated dienes, lipid hydroperoxides, MDA, 4-hydroxyalkenals and Schiff's bases. These measurements were used to estimate the extent of oxidative damage.

4.4.3.1 Conjugated dienes

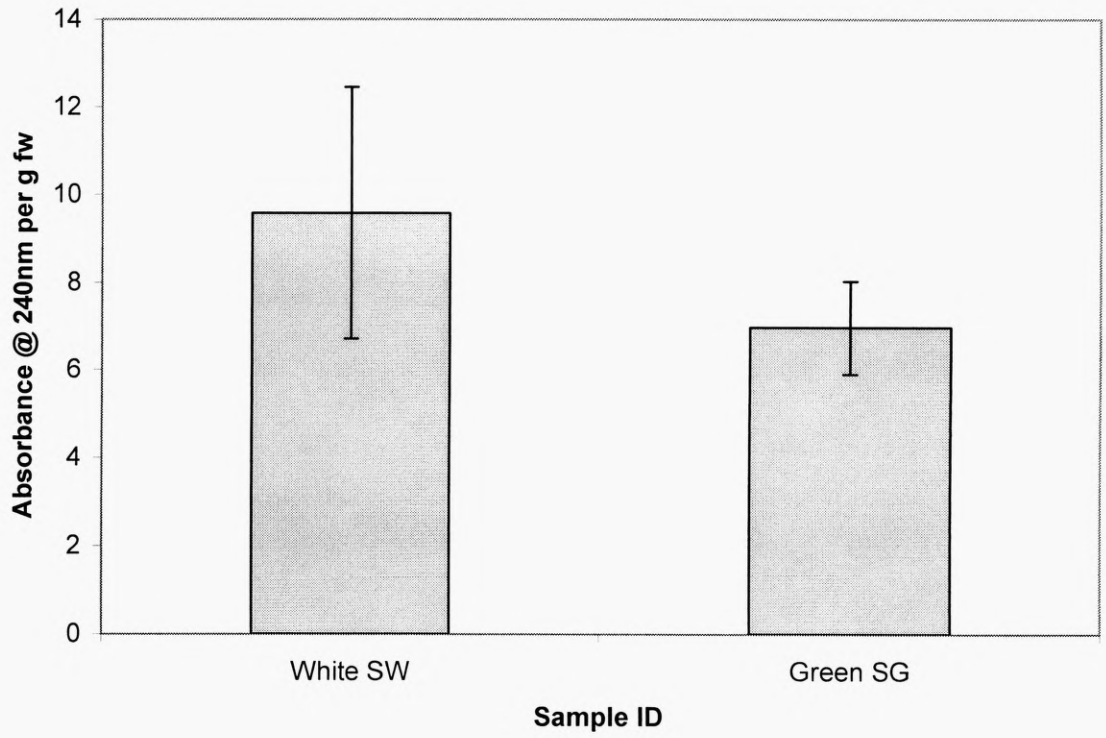


Figure 4.20: Conjugated dienes in *G. max*, White (SW) and Green (SG). Data points were calculated as the means of replicate samples, where N=9. Levels of conjugated dienes are expressed as the absorbance at 240nm and are based on one gram of fresh weight material. Errors are derived from the standard deviations of the data series.

There was no significant difference in the levels of conjugated dienes in the two *G. max* cell lines ($P>0.05$, $F=1.42$), the data was analysed using one-way ANOVA and the series satisfied all ANOVA assumptions.

4.4.3.2 Lipid hydroperoxides

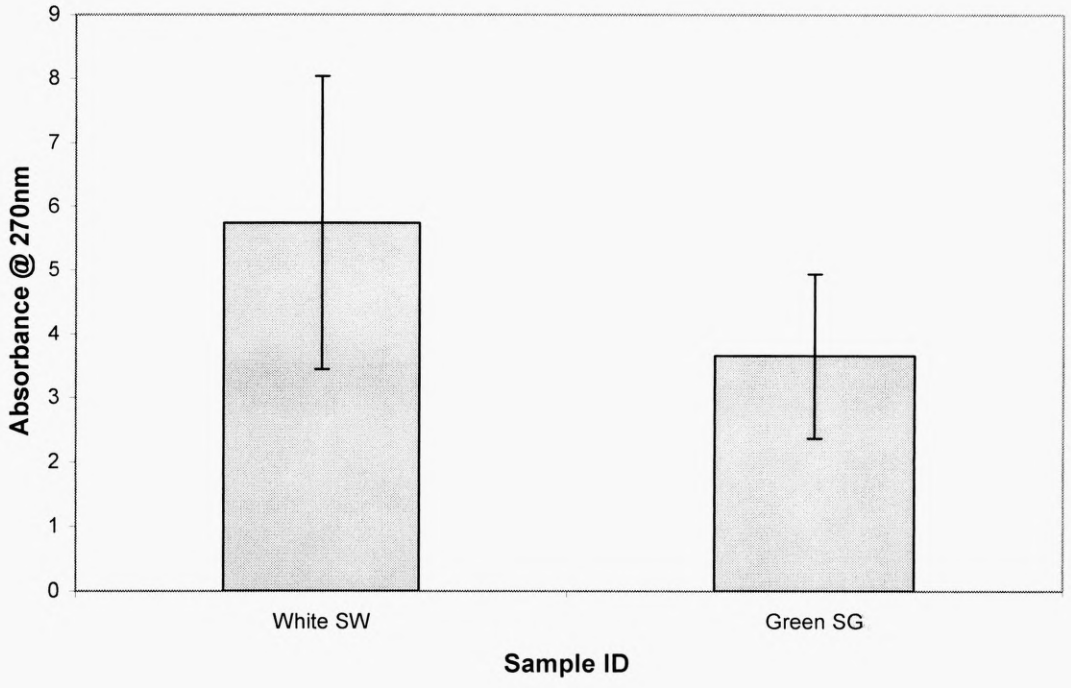


Figure 4.21: Lipid hydroperoxides in *G. max*, White (SW) and Green (SG). Data points were calculated as the means of replicate samples, where N=9. Levels of conjugated dienes are expressed as the absorbance at 270nm and are based on one gram of fresh weight material. Errors are derived from the standard deviations of the data series

The data showed no significant difference in lipid hydroperoxides in the two cell lines ($P>0.05$, $F=2.09$). ANOVA one-way analysis was used to analyse the data, which satisfied all ANOVA assumptions.

4.4.3.3 Malondialdehyde and 4-hydroxyalkenals assay (LPO-586 assay)

Total MDA + 4-hydroxyalkenals

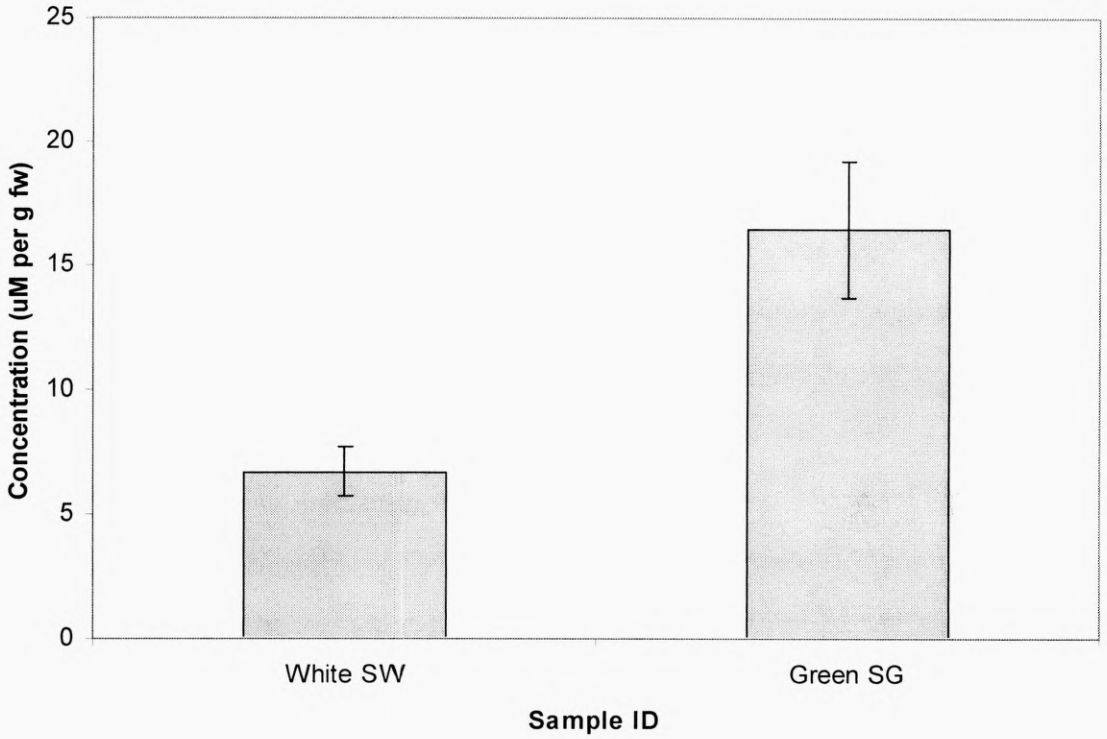


Figure 4.22: Total MDA and 4-hydroxyalkenals concentrations in *G. max,* White (SW) and Green (SG). Data points were calculated as the means of replicate samples, where N=9. Concentration is expressed in micromoles and is based on one gram of fresh weight material. Errors are derived from the standard deviations of the data series.

There was a significant difference between the two cell lines ($P<0.01$, $F=109.23$). The concentration of the total MDA and 4-hydroxyalkenals was significantly higher in the pigmented cell line (SG) and ANOVA assumptions were satisfied for this data series. Although ANOVA assumptions were satisfied the Kruskall-Wallis test was also applied, as the data for the MDA-only assay was non-parametric, therefore for comparison the same statistical test was used for both. Using the Kruskall-Wallis test it was confirmed that there was a significant difference between the two cell lines

(P<0.01, H=12.79). Note the formation of an unidentified orange chromophore was also seen in these *G. max* cell lines (see section 3.4.3.3).

MDA

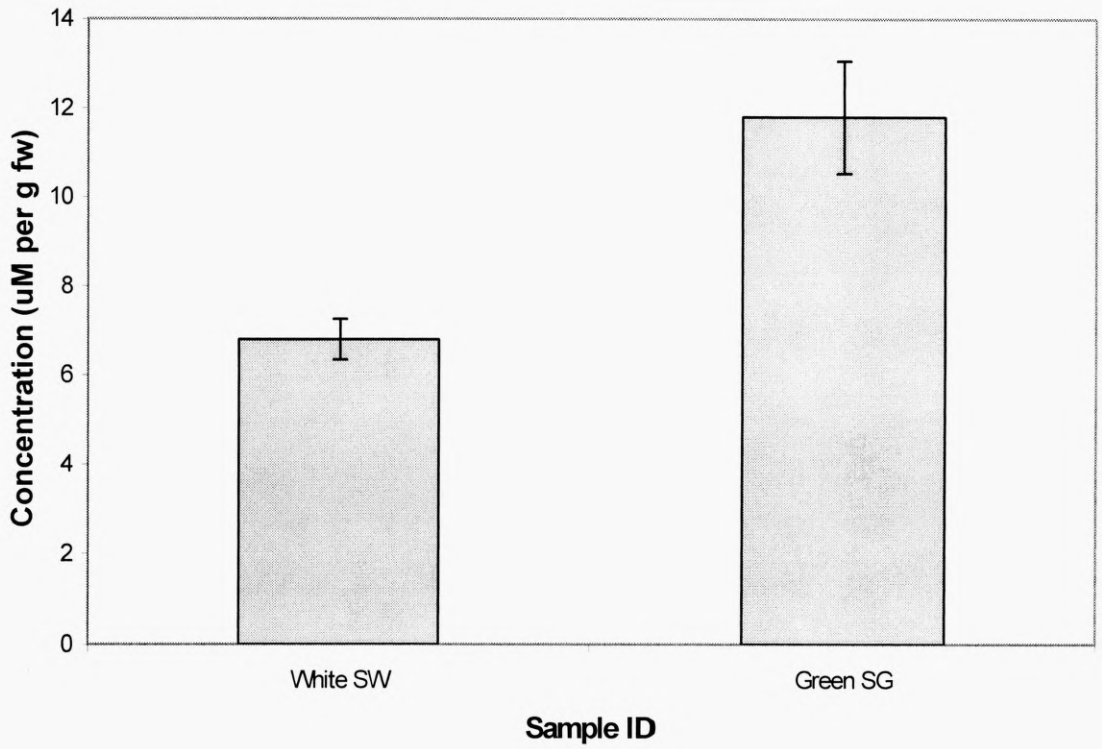


Figure 4.23: MDA levels in *G. max*, White (SW) and Green (SG). Data points were calculated as the means of replicate samples, where N=9. Concentration is expressed in micromoles and is based on one gram of fresh weight material. Errors are derived from the standard deviations of the data series.

The two *G. max* cell lines were significantly different, the pigmented line (SG) had a significantly higher concentration of MDA and the Kruskall-Wallis test was used as the data was non-parametric (P<0.01, H=12.79).

Comparison of MDA and 4-hydroxyalkenals

To determine the level of 4-hydroxyalkenals (4-Hae) the MDA value was subtracted from the total aldehydes value and Figure 4.24 below shows the ratio of MDA to 4-hydroxyaldehydes.

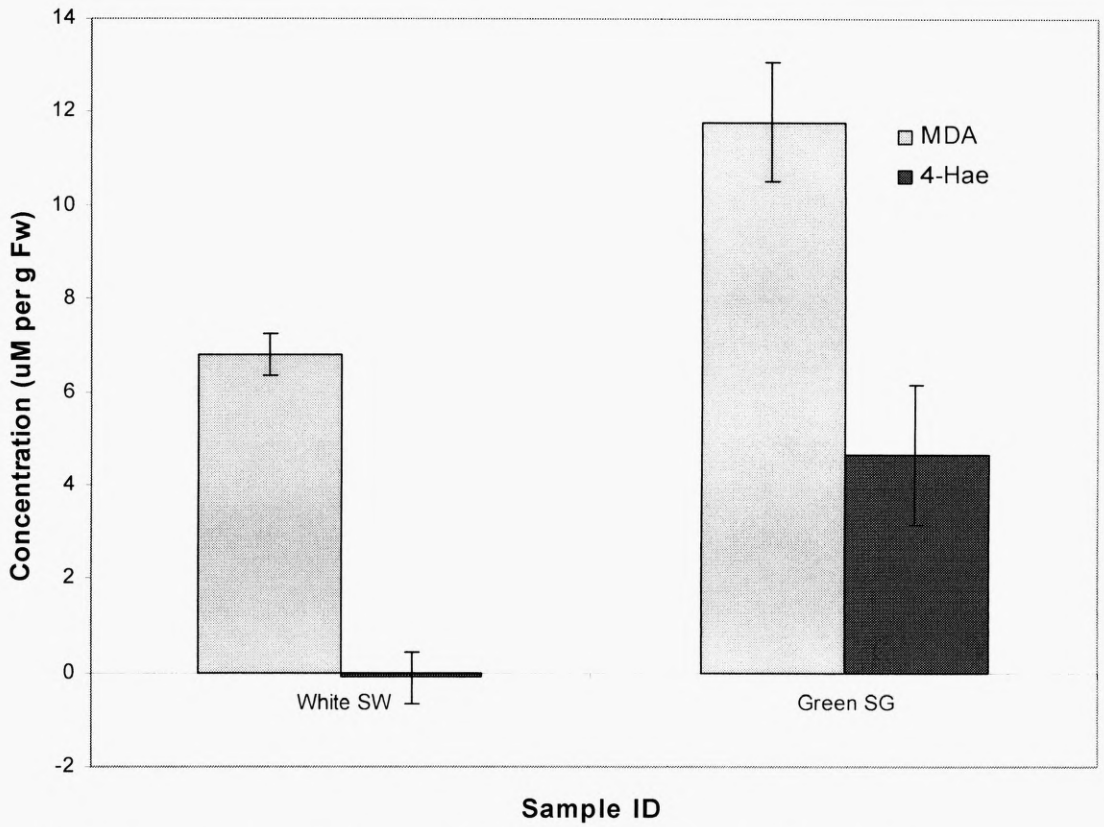


Figure 4.24: Comparison of MDA and 4-Hae in *G. max*, White (SW) and Green (SG). Data points were calculated as the means of replicate samples, where N=9. Concentration is expressed in micromoles and is based on one gram of fresh weight material. Errors are derived from the standard deviations of the data series.

Figure 4.24 shows that the pigmented line (SG) has higher levels of MDA and 4-hydroxyalkenals compared to the non-pigmented cell line (SW). In the white cell line (SW) there were no detectable 4-hydroxyalkenals. However, as with the *Beta vulgaris* samples an interfering, unidentified orange chromophore, of unknown origin, with a maximum absorbance at 469nm. This was formed on reaction with the assay

reagents, which are most likely interfering with the assay (see Appendix 6.2), making the results difficult to interpret accurately.

4.4.3.4 TBARS assay

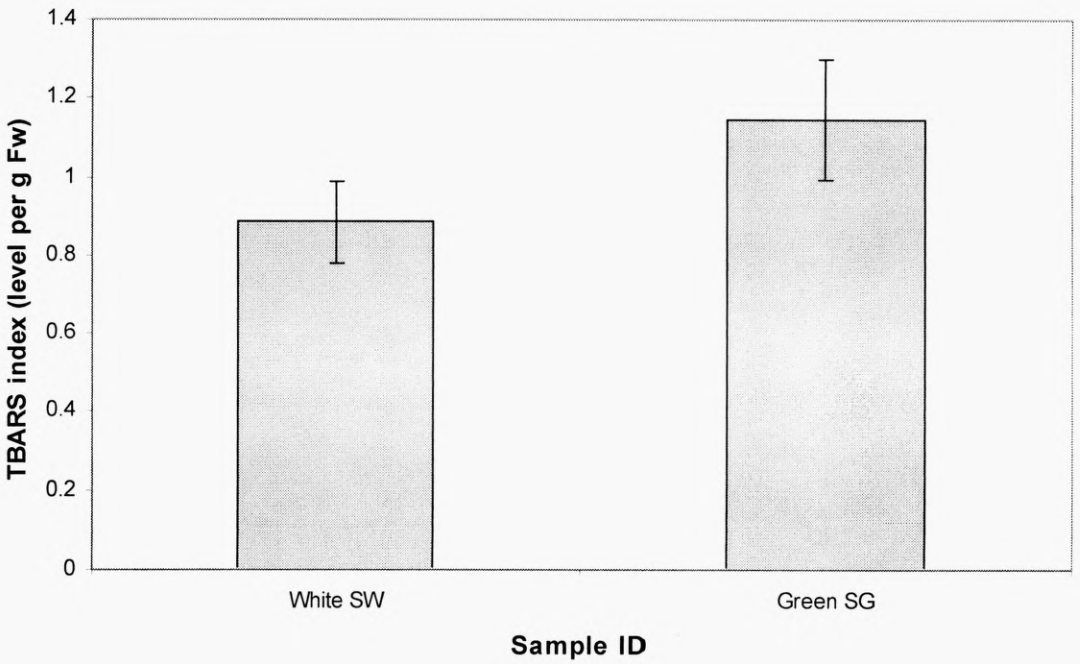


Figure 4.25: TBARS reactivity in *G. max*, White (SW) and Green (SG). Data points were calculated as the means of replicate samples, where N=9. Reactivity is expressed as the absorbance at 532nm and is based on a gram of fresh weight material. Errors are derived from the standard deviations of the data series.

The data series satisfied all ANOVA assumptions and there was a significantly higher level of TBAR reactive species in the green-pigmented (SG) cell line ($P<0.05$, $F=7.02$). The data for this assay are reflected by those of the LPO-586 assay.

4.4.3.5 Schiff's bases

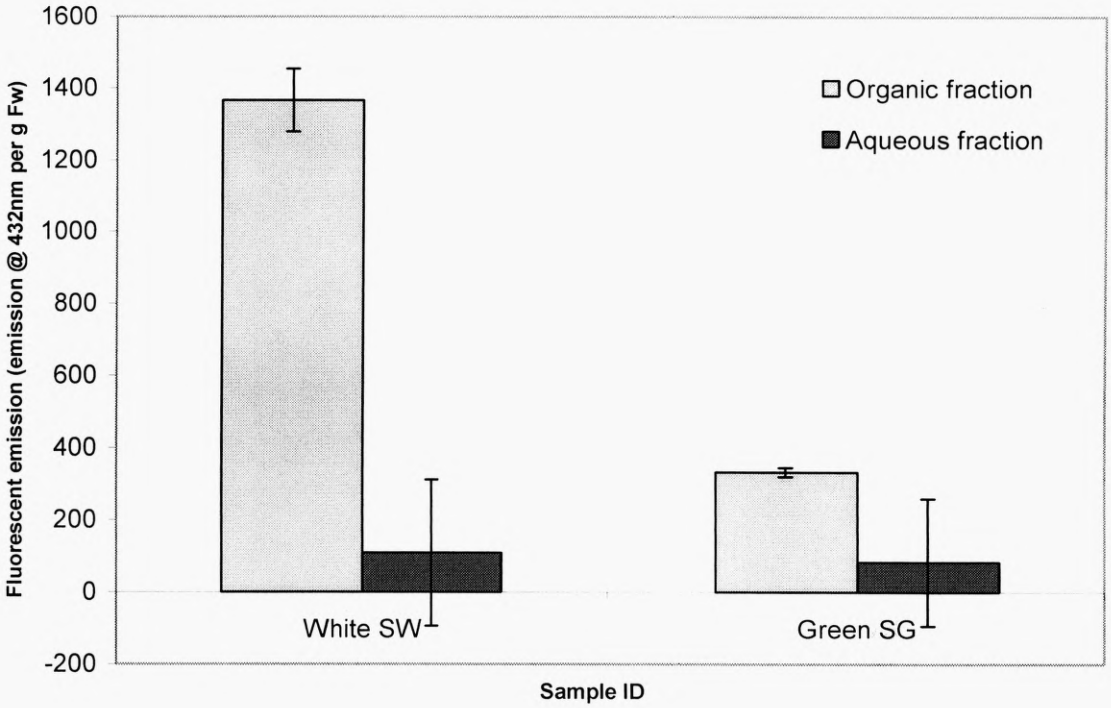


Figure 4.26: Schiff's bases in *G. max*, White (SW) and Green (SG). Data points were calculated as the means of replicate samples, where N=9. Concentration is expressed in the organic and aqueous fraction as the fluorescence at 430nm and is based on one gram of fresh weight material. Errors are derived from the standard deviations of the data series.

There was a significant difference in the level of Schiff's bases in the organic fraction ($P<0.05$, $H=7.29$), however no significant differences were found in the aqueous fraction ($P>0.05$, $H=0.07$). The data series in both cases were non-parametric therefore the Kruskall-Wallis test was used for statistical analysis. And show that both cell lines contain very little Schiff's bases in the aqueous fraction, but a marked increase in Schiff's bases in the organic fraction in white cell line (SW).

4.5 DISCUSSION: A STUDY OF *IN VITRO* AGEING IN *G. MAX* CULTURES

Two aged, cell lines were examined, one pigmented (SG) and the other non-pigmented (SW). The physical attributes of pigmentation has differentially been arrested suggesting that these lines have different metabolic profiles and capacities. It is known that pigmentation can impact ROS status through altered susceptibility to photo-oxidation (Benson and Noronha-Dutra, 1984 and Benson and Bremner, 2004). By characterising the ability of antioxidant systems to remove ROS and the extent of oxidative damage in these cells lines it may be possible to elucidate the mechanisms that disturbs normal primary and secondary metabolism in aged (16-20 years old within the life time of the project) pigmented and non-pigmented cultures.

4.5.1 Consideration of pro and anti-oxidant status: in vitro implications

Callus form is similar in both cell lines except for pigmentation. Both produce a dedifferentiated mass of cells in the presence of callus maintenance PGRs (2,4-D and NAA) in the medium. Cell line (SW) is a white callus and the cells are dry and compact and tending towards friability, however despite the absence of chlorophyll, this cell line is extremely photosensitive and therefore grown in the dark to prevent extensive necrosis. A lack of carotenoids (which are photoprotectants) may suggest that this cell line has defective chloroplasts protection. However pigment and photosynthetic analysis was not a component of this study as the cultures were grown heterotrophically. Chloroplasts contain key antioxidants, (SOD and ascorbate) and an

increase in chloroplasts that are abnormal may impact global protection. The green cell line (SG) can be grown directly under a light stack and the chlorophyll intensity increases with the subculture cycle, which may represent a maturation process. This cell line also showed limited symptoms of necrosis, therefore it was not considered to be photosensitive (a symptom of malfunctioning chloroplasts). The cells from this green cell line were also small, compact and dry in texture and tending towards friability.

Growth profiles were measured for each cell line and there was no significant difference between them. A more detailed examination (see Figures 4.3 and 4.4) of both lines using a light microscope (LMx100) and an FDA staining technique identified some differences between the cell lines. Cell elongation was more frequently observed in the white cell line (SW) but was observed in both lines (see Figure 4.3). In general, the cells in the green line were smaller and more spherical and there was more interference from chlorophyll pigments (which autofluoresce under U/V light) in the green cell line, producing a red colouration. Both cell lines had granular material that appeared to accumulate around the inside of the cell membrane. Small areas of the cell appeared to fluoresce brightly from the nucleus (see Figure 4.4) in both SW and SG cell lines.

4.5.2 Pro-oxidant status and differential ageing profiles

Determining the activity of some of the key antioxidant enzymes and the levels of biomolecules involved in detoxification processes allows the determination of how

well these aged cell lines are equipped to deal with oxidative stress. A summary of the data profiles is shown in Table 4.5.

4.5.2.1 Summary of enzymatic and non-enzymatic antioxidants profiles in the cell lines of *G. max*

Antioxidant	**White** *G. max* **(SW)**	**Green** *G. max* **(SG)**
Catalase Enzyme activity per mg protein	0.00069^{c}	0.00089^{c}
Peroxidase Enzyme activity per mg protein	$0.0094^{\downarrow b}$	0.022
Cu,Zn – SOD Enzyme activity per mg protein	0	$250.84^{\uparrow a}$
Glutathione reductase Enzyme activity per mg protein	0.00634^{c}	0.0138^{c}
Glutathione S-transferase Enzyme activity per mg protein	0.00021^{c}	0.00035^{c}
Total glutathione mM per gfw	1.237	$0.865^{\downarrow b}$
Oxidised glutathione mM per gfw	0.641^{c}	0.433^{c}
Total ascorbate µM per gfw	21.10	$29.04^{\uparrow b}$
Reduced ascorbate µM per gfw	5.79	$8.18^{\uparrow a}$
Protein-bound Sulphydryl µM per gfw	2.14	2.42

Table 4.5: Summary of the absolute values obtained from the biochemical analysis of the key antioxidants in the *G. max* cell lines. Statistical differences between the cell lines are marked as follows: ↑a or ↓a = very highly significant, ↑b or ↓b = significant, c = no significant differences, the direction of the arrow denotes whether it is a higher or lower value.

Superoxide dismutase, catalase and peroxidase

The leakage of electrons from the electron transport chain results in the production of superoxide radicals. Superoxide radicals are transformed by superoxide dismutase, into hydrogen peroxide, which is then converted to water by catalase and peroxidase (see Figure 3.29). As superoxide radicals, are uncharged molecules they cannot diffuse through cell membranes, therefore it is essential that these radicals are

detoxified *in situ* and before they undergo local damage. Superoxide dismutase is located in the same place as the electron transport chains, i.e. in the chloroplasts and the mitochondria. In plants Cu,Zn-SOD's are generally found in the chloroplasts and Mn-SOD's in the mitochondria.

The majority of total superoxide dismutases in plants are of the Cu,Zn type and the assay used during this study determined the level of Cu,Zn-superoxide dismutases. No Cu,Zn-SOD activity was detected in the white cell line (SW) but SOD activity was very high in the green cell line (SG). The presence of Cu,Zn-SOD in the green cell line does suggest that both these *G. max* cultures may have had Cu,Zn-SOD activity at some point, but the white cell line appears to have lost its activity, which may be connected with the lack of intact chloroplasts. A decrease in Cu,Zn-SOD activity will cause superoxide radicals to accumulate in the cell and if other forms of SOD i.e. Mn-SOD or Fe-SOD, are unable to compensate the superoxide radicals are capable of initiating several free radical chain reactions and lipid peroxidation. Another consequence of lowered SOD activity is that there may be a corresponding decrease in hydrogen peroxide levels. Although widely recognised as a ROS, there is increasing evidence to support the role of hydrogen peroxide as an important signalling molecule. Below a critical concentration, hydrogen peroxide is thought to play a role in signalling growth and development responses (Kairong *et al.*, 2002) but above that concentration hydrogen peroxide acts as ROS and can cause oxidative damage.

The white cell line (SW) also had very low catalase and peroxidase levels (see Figures 4.4.1.3 and 4.4.1.4) which leave the cell vulnerable to hydrogen peroxide toxicity However as this cell line has no detectable Cu,Zn-SOD then this may mean that there

is less hydrogen peroxide being produced. However, there are still several other peroxidase reactions that can produce hydrogen peroxide. Cu,Zn-SOD is often found in very high concentrations in plant chloroplasts and the data collected from these two cell lines suggests that the cell line (SW) that lacks any chlorophyll (which may suggest ruptured chloroplasts), had no detectable Cu,Zn-SOD. Therefore, there may be a possible link between the disruption of the chloroplasts and the loss of Cu,Zn-SOD activity; however unless chloroplast studies are carried out this cannot be confirmed.

Another possibility is that the lack of Cu,Zn-SOD is caused, along with the lack of chlorophyll expression, by somaclonal variation, DNA mutation or multiple mutations coding for both pigments and SOD enzyme expression. This may be explored in further studies through gene expression analysis and the determination of which isoforms of SOD are active and where they are located. This work could not be undertaken during the time course and remit of this study. The loss of Cu,Zn-SOD located in the choroplasts may not substantially effect the cell if primary energy production is through respiration in the mitochondria. However, if Mn-SOD was also deactivated in this cell line then the mitochondria may have no protection against *in situ* superoxide production and this will adversely affect the cell.

Catalase is the primary enzyme involved in the removal of hydrogen peroxide and in both the green and white *G. max* cell lines catalase activity was extremely low, which will result in the accumulation of hydrogen peroxide in the cells. As Cu,Zn-SOD is still active in the green cell line and producing hydrogen peroxide via superoxide dismutation, the hydrogen peroxide levels on a whole may be expected to be higher in

the green cell line. Catalase is vulnerable to ROS deactivation due to its structure (Boon, *et al.*, 2001) therefore it is highly likely that in these aged cell lines catalase would by the first enzyme to be decreased as a result of oxidative stress, in addition there is evidence to suggest that catalase is not as active during long term tissue culture and in particular callus cultures (Benson and Roubelakis-Angelakis, 1994). Peroxidase is also capable of removing hydrogen peroxide but that is not the only role for peroxidase and in some cases peroxidase reactions can, in fact, produce hydrogen peroxide. Peroxidase activity was significantly higher in the green cell line (SG). This is the only cell line that exhibited any Cu,Zn-SOD activity, thus producing more hydrogen peroxide therefore does the higher peroxidase level perhaps compensate for the lack of catalase? The other possibility is that higher peroxidase values may be linked to increased lignin synthesis; however in callus cultures lignin content is usually low.

If hydrogen peroxide is not detoxified and is allowed to accumulate in tissues, then it is likely to take part in the Haber-Weiss and Fenton reactions (see section 1.9.3). These reactions give rise to hydroxyl radicals, which are capable of causing extensive cell damage as a result of lipid peroxidation, uncoupling of the redox regulation and modification of individual DNA bases that can lead to mutations that cause non-reversible damage and phenotype changes. Catalase and peroxidase activities in both cell lines were very similar and catalase activity extremely low, which will undoubtedly affect the ability of the cells to remove hydrogen peroxide. Superoxide dismutase activity was different in both cell lines. The lack of Cu, Zn-SOD in the white cell line this will expose this culture to damage from superoxide radicals, whereas in the green cell line with high SOD activity, the damage is more likely to be

as a result of hydrogen peroxide, due to the low levels of catalase. Examination of the some of the other antioxidants will provide further insight to the extent of oxidative stress and overall antioxidant status in each cell line.

The increased peroxidase levels in the green cell line suggest that it has the greater capacity to detoxify hydrogen peroxide, which is important as this cell line has significantly higher Cu,Zn-SOD activity and would be expected to produce more hydrogen peroxide than a cell lacking Cu,Zn-SOD. Higher guaiacol peroxidase activity is also correlated with greater lignin production (Quiroga *et al.*, 2000), therefore increased peroxidase may also indicate that this cell line has a higher lignin content, which will give the cell increased rigidity and possibly make this cell line less prone to cell lysis. This observation might explain why there may be more intact chloroplasts present in the green cell line; however lignin production is normally increased in developing tissues. Therefore as these cell lines are both dedifferentiated higher lignin content is unlikely to be the sole reason for increased peroxidase activity.

4.5.2.2 Impact of the glutathione-ascorbate redox cycle, antioxidant protection and detoxification in aged cultures

Glutathione and ascorbate are both capable of taking part in several ROS detoxification reactions. In their reduced forms they are highly effective antioxidants and are capable of detoxifying a wide range of ROS, including hydrogen peroxide, superoxide radicals and singlet oxygen. In normal, healthy cells the percentage of reduced glutathione is normally around 90% (Foyer *et al.*, 2001) this value is essential

to maintain the strong reducing environment and redox gradient that drives many biosynthetic and detoxification reactions.

Both *G. max* cell lines had significantly reduced redox ratios. The white (SW) and green (SG) cell lines had a glutathione status of 0.48 and 0.50 respectively; meaning that in both lines the percentage of reduced glutathione had dropped from 90% to around 50% (see Figure 4.11). This significant decrease in reduced glutathione will have serious implications in many of the normal cell biosynthetic and detoxification reactions. Large changes in reduced glutathione will alter the antioxidant/ pro-oxidant balance of the cells and can push the equilibrium towards the pro-oxidant side promoting the formation of more free radical and secondary ROS. Also, a corresponding decrease in detoxification reactions and normal synthetic reactions may result, as many of them require a reduced environment for the reactions to take place.

During the initial onset of oxidative stress, the primary antioxidant enzymes SOD, catalase and peroxidase would normally remove the majority of the ROS or convert them into less damaging species for easier removal. Glutathione is capable of direct detoxification of a number of different ROS and will remove any excess ROS, which are left in the cell particularly when the redox potential is very high, as this type of reaction is highly favoured. During prolonged oxidative stress due to *in vitro* ageing it is clear from this study that the activity of the primary antioxidants (SOD, catalase and peroxidase) decreased significantly, most likely as a result of ROS induced enzyme deactivation. Therefore there is an added requirement for non-enzymatic antioxidants such as glutathione and ascorbate to remove excess ROS.

Glutathione, like any other antioxidant, can be depleted during long-term oxidative stress as a result of demand outweighing supply. The rapid increase in glutathione oxidation should result in a corresponding increase in glutathione synthesis or recycling. However, from the significantly decreased percentage of reduced glutathione it would appear that this is not happening in either of these cell lines. In the recycling and synthesis process there are two major factors to consider that may explain the lack of glutathione replacement. Firstly, it could be due to ROS-induced deactivation of enzymes required in either the synthesis or recycling processes or, secondly, a reduction in substrate availability required in both reactions. Removal of ROS is not the only process in which glutathione participates. Reduced glutathione also conjugates toxic compounds via glutathione S-transferase, and assists the replenishment of protein-bound sulphydryl groups, as well as playing a crucial role in the Halliwell-Asada cycle involved in recycling ascorbate (see Figure 3.30).

The role that ascorbate plays in detoxification processes is not entirely clear, though it has been established that ascorbate, like glutathione, is capable of the direct detoxification of several ROS, including hydrogen peroxide, superoxide radicals and singlet oxygen (Anderson, 1985, Cotgreave and Gerdes, 1998 and Dickinson and Forman, 2002). However, its response in removing these ROS is not always directly correlated with higher levels of oxidative stress. In some cases ascorbate activity was shown to be at its highest during key growth and developmental stages with a move towards the reduced side (around 90% of total ascorbate in reduced form), (Noctor and Foyer, 1998 and Tommasi *et al.*, 2001) suggesting that ascorbate is a very important antioxidant that provides added protection to prevent any ROS damage

during key developmental stages. This is to be expected as enhanced oxidative metabolism will accompany growth changes and accelerations.

Efficiency of ascorbate protection, like glutathione, is contingent upon the ratio which is dependent on the rate of synthesis and the rate of recycling, which again depends on the enzymes involved and substrate availability. The ratio of reduced and oxidised ascorbate was almost identical in both cell lines at approximately, both cell lines had around 35% of the total ascorbate in its reduced form, which means that there was a much higher percentage of dehydroascorbate in both cell lines. Dehydroascorbate is effectively a stored form of ascorbate, however as suggested, previously ascorbate may only play an active role during differentiation and key developmental stages. As both these cell lines are dedifferentiated then ascorbate may not play a highly active role in ROS detoxification, which may account for the fact that the majority of ascorbate is in its oxidised or "stored" form. Another possibility is that ascorbate has been oxidised in these cell lines, but the enzymes involved in recycling ascorbate may be less active due to ROS damage, therefore there is a decrease in reduction. A further limiting factor may be the shortage of reduced glutathione and NADPH, which are substrates required in the recycling reaction.

Glutathione reductase is the enzyme involved in the recycling of glutathione. It enzymatically catalyses the conversion of oxidised glutathione back to its reduced form and plants that over-express glutathione reductase have increased tolerance to photoxidative stress. This was demonstrated by using transformed species that had been genetically engineered to over-express the glutathione reductase enzyme (Foyer *et al.*, 1995). In cotton, during the initial oxidative stress, glutathione reductase

activity is increased (Meloni *et al.*, 2003). The findings from this current study show glutathione reductase activity was extremely low in both the *G. max* cell lines, suggesting that perhaps during prolonged oxidative stress, thought to be associated with *in vitro* ageing, increased glutathione reductase activity cannot be maintained. The decrease may be caused by ROS induced enzyme deactivation, reduced substrate availability, a change in gene expression or accumulation of GST conjugates, which are known to have an inhibitory effect on glutathione reductase, glutathione S-transferase and NADPH facilitating enzymes (Foyer *et al.*, 1997). With such low glutathione reductase activity the emphasis on repairing the redox potential will be shifted to the synthesis pathway, however the present ratio suggests that this process is also unable to keep up with the demand for reduced glutathione. So, is the significant decrease in reduced glutathione a question of demand out-weighing supply or is it that the enzymes involved in the synthesis and recycling are deactivated by ROS? The most likely answer to this question is probably a combination of both, this area would benefit from further research to determine exactly what factors effect the decrease in GSH ratio.

Glutathione S-transferase (GST) catalyses the conjugation of glutathione to a number of electrophilic compounds, such as MDA and 4-HNE, which are toxic products produced by lipid peroxidation. GST activity has been shown to increase rapidly in response to increases in lipid peroxidation (Fukuda *et al.*, 1997). In the present work GST activity was found to be very high in both the *G. max* cell lines, suggesting that significant lipid peroxidation and the production of secondary products was taking place. If glutathione conjugates are allowed to accumulate in the cell they can become potent inhibitors of the glutathione metabolising enzymes, glutathione S-

transferase and glutathione reductase, therefore it is important that the conjugates are removed quickly from the cell. Efficient removal of glutathione conjugates is dependent on the rapid transport to the vacuole through glutathione pumps located in cell membranes. And, if this is the case a further compromise to membrane damage by ROS and lipid peroxidation is inevitable and manifests the cause and effect component implicit to oxidative stress. Glutathione reductase activity in both *G. max* cell lines was also extremely low, which may suggest that the accumulation of conjugates might be responsible for inhibiting glutathione reductase activity. On the other hand, it would appear from the high level of glutathione S-transferases, either glutathione S-transferase is not as susceptible to inhibition from GS-conjugates, or the high presence of glutathione S-transferase may be caused by the lack of reduced GSH required to carry out the conjugation.

Sulphydryl groups are compounds containing –SH groups and they can be divided into two types, protein bound and non-protein bound. Protein bound sulphydryls are attached to several protein and membrane structures and are important in maintaining the integrity of cell membranes. The oxidation of –SH groups can result in membrane instability, changes in ion channels and can lead to membrane peroxidation. The oxidation of sulphydryl groups attached to enzymes can result in enzyme deactivation. They offer protection to the cell components and are effectively preferential sites of oxidation, therefore have been described as antioxidants in there own right (Hu *et al.*, 1993).

The present data shows that levels of protein bound and non-protein bound SH were almost the same in both cell lines and there was no significant difference between

them (see Figure 4.17). Protein bound sulphydryl group content in both cell lines were relatively low compared to other aged species (*Beta vulgaris,* cell line N), however they were not as low as the level observed in the cancerous *Beta vulgaris* cell lines (see previous chapter) . The majority of the non-protein bound sulphydryl is comprised of reduced glutathione (GSH), which has already been shown to be significantly reduced in both cell lines (see reduced glutathione, Figure 4.9). The low level of protein bound sulphydryl groups is caused by the increased rate of oxidation and the decreased rate in sulphydryl replacement, which will undoubtedly be a result of the decrease in available reduced glutathione, which is essential to this reaction. Therefore, lowered membrane bound proteins will lead to increased lipid peroxidation and increased enzyme deactivation as a direct result of membrane oxidation.

4.5.2.3 Consequences of antioxidant compromise: Reactive oxygen species (ROS)

The measurement of ROS and lipid peroxidation products will highlight the extent of oxidative damage caused as a result of *in vitro* ageing. Measuring ROS levels can be difficult without expensive equipment and complicated assays. A simple assay was used during this study in an attempt to measure hydrogen peroxide levels in cells; the assay chosen is used in mammalian studies and was adapted for use with *in vitro* plant cultures (Packer, 1984) and was sensitive between 50-200µL per mL (based on a standard curve made between this range using 30%, v/v hydrogen peroxide). Even though the standards for this assay gave an accurate line of fit and were highly reproducible, no hydrogen peroxide was detected in any of the *G. max* cell lines. The reasons for this as explored in the previous chapter are applicable here (see section 3.7.2.3).

Dimethyl sulphoxide (DMSO) can be used as a radical trap to quantify the levels of hydroxyl radical and this assay has been successfully utilised in plant species previously (Fleck *et al.*, 2000). However during this current study no hydroxyl radical activity was detected in either of the two *G. max* cell lines. The reasons for this are most likely the same as discussed in the previous chapter (see section 3.7.2.3) and see sections 3.6.2.2 for alternative hydroxyl radical assays.

4.5.2.4 Secondary oxidative stress and lipid peroxidation

Summary of lipid peroxidation profiles in *in vitro* aged *G. max*

Peroxidation Product	**White** *G. max* **(SW)**	**Green** *G. max* **(SG)**
Conjugated dienes Abs at 240nm	9.57^{c}	6.96^{c}
Lipid hydroperoxides Abs at 270nm	5.74^{c}	3.65^{c}
TBARS TBARS index	0.88	$1.14^{\uparrow b}$
Schiff's bases Organic Abs at 430nm	$1365.92^{\uparrow b}$	333.46
Schiff's bases Inorganic Abs at 430nm	109.21^{c}	82.86^{c}

Table 4.6: Summary of the absolute values obtained from the biochemical analysis of the lipid peroxidation products in the *G. max* cell lines. Statistical differences between the cell lines are marked as follows: ↑a or ↓a = very highly significant, ↑b or ↓b = significant, c = no significant differences, the direction of the arrow denotes whether it is a higher or lower value.

Lipid peroxidation products were measured to determine the extent of peroxidation in the two *G. max* cell lines. Conjugated dienes and lipid hydroperoxides were the first two products formed during the peroxidation process and the levels of there two ROS species were lower in both cases in the green cell line (SG), however the differences were not statistically significant. The levels of primary lipid peroxidation products suggest that the extent of lipid peroxidation is similar in both cell lines. Secondary

products of lipid peroxidation include MDA and 4-hydroxyalkenals and they were measured using the LPO-586 assay. This assay presented problems as, unexpectedly, there was a bright orange compound formed (see Appendix 6.2). This unidentified orange chromophore was also observed in the *Beta vulgaris* samples and rendered the results invalid, suggesting that there may be possible problems with the use of this specific assay in plant species, in particular aged plant species (see section 3.7.2.4).

The TBARS assay demonstrated that TBA reactive substances were significantly higher in the green cell line (SG). During this assay, like the LPO-586, assay there was a slight indication of an orange coloured compound forming in all the samples, but the colour was not as intense as in the LPO-586 assay. These orange coloured compounds have been reported previously during the TBARS assay and were identified as other lipid peroxidation products (dienals, 2,4-dienals and various other saturated aldehydes), which are not thought to affect the absorbance reading of this particular assay (Kosugi and Kikugawa, 1989). The final lipid peroxidation product measured was Schiff's bases, which are long term oxidative stress indicators, as they form permanent bonds with amino acids. Schiff's bases can also be considered as age pigments as, generally, they increase with age. The results showed that the levels of Schiff's bases were significantly higher in the white cell line (SW). This may suggest that although ROS damage and oxidative stress is, at this point very similar in both cell lines it may have been occurring in the white cell line from an earlier age, hence the marked increase in Schiff's bases.

4.6 CONCLUSIONS

- These cell lines resembled normal callus in that they are PGR dependent and proliferate in a chaotic and unorganised manner. The only immediate visible pronounced difference is the colour, which is caused by the lack of chlorophyll in the white cell line (SW).
- Both *G. max* cell lines experience elevated oxidative stress, with both cell lines exhibiting significantly decreased activities of catalase and peroxidase
- Peroxidase activity was considerably lower in the white cell line, suggesting that this line may be even less well equipped to deal with hydrogen peroxide, however the marked differences in Cu,Zn-SOD activity were a significant discovery.
- In the white cell line, no Cu,Zn-SOD activity was detected. The activity of Cu,Zn-SOD in the green cell line may be due to the higher levels of functional chloroplasts (represented by chlorophyll production).
- Another possibility is that for the white cell line, all Cu,Zn-SOD has been deactivated via reaction with ROS, or expression altered due to changes in DNA. Suggesting that the white cell line may have undergone major changes in enzyme expression, which may have long-term consequences on its defence against ROS.
- Reduced glutathione in both cell lines was extremely low resulting in a glutathione ratio far below the expected. Normal cells have 90% or over of their total glutathione reduced and in these cell lines this figure was diminished to around 50%. Suggested routes of GSH depletion are as follows:
    - Glutathione is depleted more rapidly as a result of an increase in the repair processes, such as protein-bound sulphydryl replacement, and ascorbate recycling.

  - Due to increased ROS there may be a reduction in the enzymes involved in the synthesis and recycling of glutathione, which may also contribute to GSH depletion.
  - The accumulation of oxidised glutathione (GSSG) is also a problem that the cell must contend with as GSSG can also take part in oxidative reactions and initiate free radical reactions.

- This decrease will have major impacts on the antioxidant / pro-oxidant equilibrium that exists in cells, pushing towards the pro-oxidant side, promoting further ROS reactions.
- Glutathione S-transferase activity was very high (0.0002-0.00035 Abs change.Min. g fw^{-1}) in both the *G. max* cell lines suggesting that levels of toxic products were higher than normal.
- After glutathione conjugated facilitated by glutathione S-transferase the resulting conjugates must be removed otherwise they can have an adverse effect on the activity of enzymes, in particular glutathione reductase, therefore the removal of conjugates is dependant on the efficiency of the membrane pumps.
- A diminution in glutathione reductase activity will also contribute to a further decrease in the production (recycling) of reduced glutathione.
- In both these cell lines the ratio of ascorbate to DHASC was extremely low 35%) and at this level, ascorbate would not be effective in any detoxification processes and/or in direct antioxidant protection.
- A reduction in protein bound sulphydryl groups can lead to destabilisation of membranes, which produces to an increase in lipid peroxidation. Also oxidation of sulphydryls attached to enzymes can lead to increased enzyme deactivation.

Primary lipid peroxidation products, conjugated dienes and lipid hydroperoxides levels did not differ between the two *G. max* cell lines. Secondary lipid peroxidation products and associated markers of LPO were detected in both cell lines and the main conclusions were as follows:

- The LPO-586 assay was invalidated as an inappropriate therefore the levels of 4-HNE were not successfully quantified, however the TBARS assay showed that there was a significantly higher TBA reactive species (which includes aldehydes formed during lipid peroxidation) in the green cell line; however the results were not vastly different.
- Schiff's bases were significantly higher in the white cell line, and overall it would appear that an overwhelming of antioxidants is accompanied by an increase in lipid peroxidation products.
- Both sets of results suggest that the decrease in antioxidants and increase in lipid peroxidation products were at similar levels in both cell lines suggesting that they both had very similar levels of oxidative stress.

So far, these profiles suggest that the failing of the primary antioxidant enzymes has a down-stream effect on the levels of other antioxidants - in particular glutathione and ascorbate. As glutathione is an important biological buffer, which regulates several antioxidant and non-antioxidant processes, the disruption of metabolic pathways in these lines may not be limited to antioxidant reactions. Decreased levels of glutathione in both lines indicate that they have a severe imbalance in redox balance which in turn pushes the antioxidant / pro-oxidant equilibrium towards the pro-oxidant side. The cell lines show no sign of recovering and not one antioxidant in particular appears capable of re-equilibrating this balance; therefore it appears that

these cell lines are "trapped" in an antioxidant decline. This results in a pro-oxidant outcome corresponding to increases in free radicals, ROS and greater lipid peroxidation. It would be expected that this may lead to an increase in the level of genetic mutations resulting in phenotype, cytological and morphological changes all are typical of the ageing process and will progress as it continues. The overall conclusion is that a combination of a decrease in antioxidant protection and the accumulation of ROS in *in vitro* aged cell lines might be a key factor in *in vitro* ageing and implicates progressive loss of tissue culture responses, such as reduction in embryogenesis, commonly associated with long-term tissue culture.

5.0 CHAPTER FIVE: GENERAL DISCUSSION

This thesis concludes with a general overview of the experimental findings related to the broader field of plant tissue culture recalcitrance, morphogenetic competence and genetic stability with respect to two "models":

(1) *In vitro* plant ageing in "ancient" soybean cultures.

(2) Sugarbeet cultures in various stages of neoplastic progression.

Both systems studied in parallel, have presented a novel opportunity to explore the role of oxidative stress and its various inter-relationships with *in vitro* plant stress, ageing and cancer. Processes that may underpin abnormal development and *in vitro* recalcitrance, particularly related to habituation. In the long-term-term, the pathways that lead to, and are a consequence of oxidative stress *in vitro,* may predispose the plant tissue culture to genetic instability. The general discussion will therefore first consider the programmed basis of plant cell dysfunction with respect to ageing and then proceed to discuss the as yet relatively under explored concept of plant cancers. Throughout the discussion the applied importance of these processes in *in vitro* plant systems and biotechnology will be considered, leading to the consideration of how the knowledge gained from this study might be exploited to assist in improving plant tissue culture performance.

5.1 OXIDATIVE STRESS AND *IN VITRO* PLANT CELL AGEING

Ageing is an inevitable biological process that affects all living organisms and has been defined as:

"a programmed *genetic process associated with morphological and functional changes in cellular and extra-cellular components aggravated by injury throughout life resulting in a progressive imbalance of the control of regulatory systems of the organism*"

(Yu, 1996).

The process of cell ageing is in itself not always responsible for the ultimate cause of cell death, but predisposes the cell to become more susceptible to disease and age-related degeneration and it is these age-related disorders that often cause death (Spiteller, 2001). Some of the most significant consequences of ageing are the loss of vigour, reproductive capability and regenerative competence. In the case of *in vitro* plant cells this can lead to the loss of totipotency and juvenile growth, and, there is evidence that the process may also predispose cells to genetic instability. Particularly if *in vitro* plant cultures are maintained in a dedifferentiated state for extended periods of time.

Totipotency is an intrinsic property of many plant cells, without which they lose their ability to regenerate to form a complete plant. It is this property that is exploited by plant biotechnologists, such that elite and/or genetically modified plant cells can be regenerated into whole plants with useful traits. Therefore, it is important to consider

the genetic stability of plants recovered and regenerated from plant tissue culture and most especially those that have been cultured for extended periods in a dedifferentiated state.

Organised plant growth occurs by differential growth and expansion initiated in defined growth centres (meristems) in a similar mechanism to stem cells. Meristems at the tip of the shoot axes are essential for the longevity of the whole plant (Thomas, 2002). These underpin new growth, however an underlying ageing process exists in all cells and which precedes senescence, death and necrosis. As long as the rate of cell death does not overtake the rate of new growth the plant will more than likely survive. But if the rate of cell death exceeds the rate of new growth then the plant will eventually die. This process is pre-determined by the natural life cycle of the *in vivo* plant and, as a consequence, plants can be categorised broadly into annuals and longer lived perennials. Indeed, death within an annual life cycle may be considered an important adaptive mechanism for the overall survival of the species. However, during ageing, meristems of long-lived species are thought to accumulate several genetic mutations, which may be caused by specific changes in coded gene expression or as a result of ROS mediated DNA damage, leading to a decrease in viability (Thomas, 2002). Thus, it is very important to consider the consequences of "releasing" the totipotent potential of *in vitro* cells that may have been altered genetically by the ageing process either *in vivo, in vitro*, or both. This possibility has been explored extensively in the phenomenon called somaclonal variation.

5.1.1 Somaclonal variation: a consideration of its role in in vitro ageing

Somaclonal variation can be described as phenotypic variation that can be either genetic or epigenetic in origin. This form of genetic variation occurs during *in vitro* tissue culture and can be inherited and is transmitted to plants regenerated from the tissue culture (Scowcroft and Larkin, 1982). It manifests itself as random mutations, which can result in changes in chromosome numbers (polyploidy and aneuploidy), changes in chromosome structure, including translocations, duplications and deletions and changes in DNA base sequences by mutations. Epigenetic changes can also occur and these include DNA amplification and methylation (Schaeffer, 1990).

Somaclonal variation is most commonly manifested in plants that are regenerated in *in vitro* tissue cultures that are disorganised and/or that have a predisposition to adventitious (i.e not from the original) meristem development. It is more frequently observed in dedifferentiated callus maintained on 2,4-D supplemented medium however it also occurs as a result of other regeneration phases (Saieed *et al.*, 1991). For example, Somaclonal variation was observed in rice (Abe *et al.*, 2002) maintained in culture over a long period of time (36 months) and using southern blotting techniques and fifteen chloroplast DNA probes it was found that large areas of the chloroplast DNA genome had been deleted. Some of the variants were capable of regenerating whole plants many of which had phenotypic differences; however some were incapable of regenerating whole plants. It was concluded that the area of the genome deleted in these variants was connected with the ability of the plant to regenerate, thus the deletion caused a loss in totipotency (Abe *et al.*, 2002).

The possibility that oxidative stress may have a role in plant ageing and plant genetic instability generated *in vitro* has been explored by Benson, Harding and co-workers over several years (Benson *et al.*, 1997 and Benson, 2000). During the *in vitro* manipulation (callus induction) of Vitis cultures there was a pronounced increase in TBARS and fluorescent oxidation products, and changes in sulphydryl status and catalase activity were also observed (Benson and Roubelakis-Angelakis, 1994). In *S. tuberosum* cultures dedifferentiation was accompanied by an increase in free radical activity, and importantly in recalcitrant species the free radical activity detected was twice that of non-recalcitrant cultures (Bailey *et al*,. 1994). Morphogenic development in *in vitro D. carota* cultures was associated with changes in the levels of lipid peroxidation products 4-HNE and MDA (Adams *et al.*, 1999 and Robertson *et al*,. 1995) and in *Oryza sativa* lipid peroxidation was higher in the cell lines that were losing or had lost embryogenic potential (Benson, 1992).

There also exists the potential for tissue culture associated oxidative stress to predispose plants to somaclonal variation (Cassells and Curry, 2001 and Jain, 2001). In *Corylus avellana* L. variations in DNA methylation patterns were associated with sequential *in vitro* sub culturing (Diaz-sala, 1995) and in *Vitis vinifera* changes in DNA methylation patterns were observed on the initiation and maintenance of *in vitro* shoot and callus cultures (Harding *et al.*, 1996). Increased oxidative stress is common in *in vitro* conditions and it may contribute to ageing observed in cells that are maintained in culture over long periods of time (Harding, 1996). Somaclonal variation being potentially induced by increased oxidative stress in aged cells may explain, in part, the alterations in phenotype and changes in totipotency (Nehra *et al.*, 1992 and Cassells and Curry, 2001).

The morphological and functional changes that take place during *in vitro* ageing can be described as a consequence of four main factors:

(1) Loss of cellular function

(2) Loss of resistance to stress

(3) Loss of ability to maintain *status quo* of normal cell functions.

(4) Loss of the ability to repair and ameliorate the impacts of deleterious damage caused by stress.

During the ageing process there are several changes that a "normal" cell undergoes; everyday "wear and tear" and oxidative stress are the first contributors to ageing, which can lead onto chronic oxidative stress, necrosis and programmed cell death (Leshem, 1992). The long-term effects of these are changes in gene expression via epigenetic DNA changes, such as methylation (Harding *et al.*, 1996) leading to modifications in phenotype or alterations in cell turnover producing cell senescence resulting, again, in changes in phenotype (Häsler *et al.*, 2003). The loss of totipotency observed in the habituated cell lines and the morphological abnormalities observed in the habituated organogenic cell line may be associated with changes in DNA methylation patterns. The methylation status of the *B. vulgaris* cell lines used during this study were investigated, however it proved technically problematic to extract sufficient DNA from the cell lines to carry out an accurate analysis. These factors pose an important question: is *in vitro* ageing caused by a decrease in the antioxidant defence mechanisms or an increase in the generation of pro-oxidants or is it a combination of both? Most likely it is both, as oxidative stress must always be considered in terms of cause and effect. The answer still is equivocal regarding the specific factors required support a molecular explanation for cell ageing. There does

however exist considerable data supporting that free radical mediated oxidative stress is involved in ageing and plant cancers (Leshem *et al.*, 1992; Munne-Bosch, 2002 and Leshem, 1987); the studies presented in this thesis support and concur with this previous evidence. Studies of ageing, neoplastic progression and habituation offer therefore an interesting system to help elucidate the broader basis of somaclonal variation and genetic instability in plants grown in tissue culture.

5.1.2 In vitro ageing in plants: the causes and consequences of oxidative stress?

In vitro plants age in the same way as mammalian cells resulting in changes in phenotype, increased necrosis, apoptosis and loss in genetic stability. Dedifferentiation in tissue culture occurs through the application of PGRs such as 2,4-D. 2, 4-D is a synthetic auxin widely used in tissue culture, however there is increasing evidence to suggest that its long-term use in medium may contribute to *in vitro* ageing, via elevated oxidative stress (Benson, 2000; Benson and Roubelakis-Angelakis 1994 and Robertson *et al.*, 1995) and somaclonal variation (Nehra *et al.*, 1992). Dedifferentiation itself is not a natural state that a plant would normally remain in for extended periods and therefore it can predispose cells to oxidative stress, which will in turn accelerate the ageing process. Specifically this is the case for the *G. max* cell lines that have been maintained on 2,4-D and NAA supplemented medium for a many years (estimated to be around 15 years) and manifest ageing symptoms such as a loss of embryogenesis and, in the white cell line, the loss biosynthetic capabilities involving pigment expression.

Analyses of antioxidants and markers of oxidative damage were used to explore if these *in vitro* aged cells are undergoing increased oxidative damage. The white cell line had very low catalase and peroxidase activity (see Figures 4.7 and 4.8) and also had no detectable Cu,Zn-SOD activity (see Figure 4.9). Catalase, peroxidase and Cu,Zn-SOD can often be considered as the first line of defence against ROS as they remove ROS, before they damage biomolecules and manifest into other ROS species (see Figure 3.29) and a decrease in activity will have implications on secondary antioxidants and other metabolic processes. The green cell line had equally low levels of catalase, slightly higher peroxidase, however there was a marked difference in the Cu,Zn-SOD activity which was still very high in comparison in the green cell line (see Figure 4.9).

Glutathione and its related enzymes were monitored in ageing rat kidneys and it was shown that the activities of glutathione peroxidase, glutathione reductase and glutathione S-transferase all decreased with age (Cho *et al.,* 2003). Glutathione and its associated enzymes are involved in detoxification and also play an important role in redox control, and the regulation of several biochemical reactions. In both the green and the white cell line there was a highly disturbed glutathione ratio, with both cells lines having only 50% of the total glutathione in the reduced state (see Table 4.4), which is significantly lower than that (90%) required for maintaining an effective redox balance (Foyer *et al.*, 2001). Changes in levels of glutathione and the activities of its associated enzymes produce alterations in the redox potential within cellular compartments and lead to changes in the antioxidant / pro-oxidant ratio (see Figure 1.20), which if prolonged will lead to higher lipid peroxidation, accumulation of secondary toxic products, a reduction in enzyme activity, membrane instability and

increased genetic mutations. A ratio reduced to this extent may be indicative of increased oxidative stress, which in turn could be conducive to *in vitro* ageing.

It is well established that cellular ageing tends to be accompanied by a general increase in lipid peroxidation products (Wickens, 2001; Benson, 2000 and Kumar and Knowles, 1993) and in both the *G. max* cell lines the levels of the majority of lipid peroxidation markers measured during this study were elevated (see Figures 4.20-4.26). Prolonged oxidative stress will result in the continued decrease in antioxidant protection, and will be manifest as a gradual increase in lipid peroxidation (Benson and Roubelakis-Angelakis, 1994; Benson *et al.*, 1992; de Zwart *et al.*, 1999; Spiteller, 2001 and Spiteller, 1996). What is important however is that the experimental evidence generated by this study suggests that the two *G. max* cell lines do have a disturbed antioxidant / pro-oxidant balance caused by a decrease in several key antioxidants and/or increased level of lipid peroxidation, which would point towards enhanced oxidative stress. Both cell lines exhibit the classic signs of ageing and have altered phenotypes (lacking chlorophyll) and reduced totipotency (incapable of embryogenic development), therefore it would appear that significant oxidative stress and in particular a decrease in the glutathione ratio is associated with their ageing processes.

Häsler *et al.* (2003) has recently suggested that long term *in vitro* cultured plant cells may be capable of undergoing spontaneous neoplastic progressions towards fully cancerous plant cells, thus becoming habituated and losing the requirement for plant growth regulators. Although the *G. max* cell lines shared many characteristics in terms of antioxidant activities (very low catalase levels, lowered glutathione ratio and

lack of Cu,Zn-SOD) they have maintained their requirement for PGRs. The level of their hormonal dependence however has not been previously or currently investigated, therefore it cannot be confirmed if these cell lines had lost sensitivity to plant growth regulators. Therefore, it may be possible that eventually their long-term maintenance in tissue culture will lead to habituation. It is highly probable that such cells lines with compromised levels of antioxidants and continually increasing peroxidation may eventually progress towards a neoplastic state. The occurrence of spontaneous habituation should also be studied in more depth in future work, to obtain greater evidence for this phenomenon.

However, it is important to clarify that oxidative damage may not be the only factor in the decline of these cell lines as continuous dedifferentiation on 2,4-D supplemented medium may also contribute to the level of somaclonal variation, which can cause genetic alterations and lead to changes conducive to ageing. Although even in this context oxidative stress may also be indirectly involved, as synthetic auxins are themselves pro-oxidants and their incorporation in culture medium can enhance lipid peroxidation and the formation of secondary aldehydic products (Benson and Roubelakis-Angelakis 1994 and Benson, 2000). Increased oxidative damage may also induce somaclonal variation and genetic mutations via direct and indirect reactions of ROS with DNA, which may lead to the changes in the phenotypes observed in the *G. max* cell lines. DNA methylation caused by epigenetic alterations in DNA may also directly change enzyme expression, regenerative capacity and phenotypic characteristics and a further in depth study should be carried out on DNA methylation patterns in the white and green *G. max* cell lines to determine whether altered methylation patterns play a role in the phenotype changes, the differences in Cu,Zn-

SOD activity or the ability of these cell lines to carry out embryogenesis, this would form an important part of the future work on these aged cell lines.

In vitro ageing poses a significant obstacle for biotechnology programmes, as it is associated with somaclonal variation, oxidative stress and genetic instability, which all potentially reduce the regenerative capacity of cells. When cells lose partial or complete totipotency (fully habituated) or become unresponsive to tissue culture manipulations (recalcitrant), they have limited use in biotechnological applications. Therefore, the more that is understood about *in vitro* ageing the more likely biotechnologists will be able to prevent or, at best delay its deleterious consequences.

5.2 Plant cancers and neoplastic progression

There are fundamental differences between animal and plant cancers as animal cancers can be divided into two distinct groups, benign and malignant. The former are considered mild tumours that are normally localised and do not invade neighbouring tissues; the latter type are aggressive tumours that are badly limited and they invade neighbouring cells in order to survive. Arguments against the concept of plant cancer were discussed by Doonan (1996), when he postulated that it was impossible for plants to get cancer as they could not form circulating metastases, which are observed in malignant animal cancers.

In response, Gaspar (1999) suggested that although plants may not form circulating metastases, they do form the plant equivalent of mammalian benign tumours. The typical trait of a plant cancer has been defined as the "acquired and hereditary capacity

for autonomous growth in the absence of exogenously supplied auxins and cytokinins" (Gaspar, 1999). When cells become fully habituated they are completely dedifferentiated and lose all totipotency. At this stage, plant cells lose their ability to form any organised or organogenic structures through their meristems and are said to have an "irreversible loss of organogenic totipotency" (Gaspar, 1999a). At this point the cells are fully habituated and are believed to have reached the endpoint in neoplastic progressions; cells in this state are highly comparable to benign cancerous animal cells and are therefore considered to be true cancerous plant cells. However, it was acknowledged by Gaspar (1998) that plants cannot form circulating metastases because plants have the unique capacity (that animals lack) to reorganise cells into organogenic structures or regenerating meristems, therefore the typical plant cancer trait has been defined as the "irreversible loss of organogenic totipotency" (Gaspar, 1998).

Prior to the 1990s the concept of plant cancer was not well accepted and was not widely researched, however over the last fifteen years the study of habituated plant cell lines, in particular these set of *Beta vulgaris* cell lines used in this study has provided an excellent experimental system to study the varying degrees of habituation and the amount of data available for evaluation of the concept of plant cancers has increased.

5.2.1 Characteristics of cancerous B. vulgaris plant cells: Past and present findings

Since 1990 there have been numerous studies conducted on these *B. vulgaris* cell lines and the characteristics of the fully habituated cell lines have been compared at

biochemical, morphological and physiological levels. Until this present study evaluations of oxidative stress in the cultures have been very limited. Importantly, many of the previous and current findings in these cell lines are highly comparable to the characteristics in animal cancer cells (see Table 5.1 for a summary). During this current research programme the morphological profiles of the cultures (see Figures 3.4 and 3.5) were very similar to those of Hagège *et al.* (1992a), whereby the fully habituated cell lines showed signs of incomplete cell walls, representative of a reduction in cell wall differentiation. The recent cytological data published by Häsler *et al.* (2003) proposes that the normal cell line (non-habituated) may have spontaneously entered early stages of neoplastic transition towards a fully habituated state as this cell line now showed several characteristics that were previously exclusive to the fully habituated cell lines (Crevecoeur *et al.*, 1992 and Hagège *et al.*, 1992a). These characteristics include formation of multiple nucleoli (and can also be indicative of polyploidy and aneuploidy) and appearance of cell surface protrusions. In terms of the present study it was therefore an important observation that these culture morphologies were retained and this concurs with the findings of other workers in the field. This provides added confidence in the resulting biochemical profiling of these unique cell cultures.

Thus, the current study showed incomplete cell walls in the "normal" cell line on microscopical observations supporting the findings of Häsler *et al.* (2003) that these cell lines may have developed some of the characteristics of the fully habituated cell lines. However, there are still a number of cytological characteristics exclusive to the fully habituated cells lines, such as extensive necrosis, loss of chlorophyll expression and lack of cell elongation. This suggests that if the "normal" cell line is entering

neoplastic progression, it may be in the very early stages and is nowhere near as progressed as the fully cancerous cell lines (N1 and N3). The data collected by Häsler *et al.* (2003) may therefore assist in the identification of the very early characteristics and perhaps the possibility of using these characteristics as potential markers, for the onset of cancer (neoplastic progressions) in plant cells. This would provide a very important diagnostic for plant biotechnologists and an alert that potentially deleterious cell dysfunction may proceed. In terms of the present study, the extensive and unique set of oxidative stress data acquired from this cell line will provide more such information, and it supports Häsler's theory of spontaneous habituation in the normal cell line. This study has therefore considerable significance for enhancing our current understanding of the field of plant cancer research.

To place the present investigation into context, there has not been a review of the biochemical properties of the *B. vulgaris* cell lines since 1992 and studies of oxidative stress in the system are to date very limited and largely based on conjecture. The results from the present study will now be compared to the previous findings and as such it may be possible to determine whether there have been any further changes in the fully habituated cell lines and the normal cell line. Specifically as very little data has been collected on the habituated organogenic cell line, determining the activity of all the main antioxidants and levels of lipid peroxidation in this culture will contribute to the elucidation of the biochemical profile of this cell line for the first time.

Morphological similarities	Biochemical similarities	Physiological similarities
Deficiency in cell wall differentiation (Crevecoeur *et al.*, 1992)	Deficiency in tetrapyrolle compounds (Hagège *et al.*, 1992c)	Independence to plant growth regulators (De Greef and Jacobs, 1979)
Deficiency in chloroplast and mitochondria differentiation (Crevecoeur *et al.*, 1992)	Accumulation of polyamines (Hagège *et al.*, 1994)	Polyploidy and aneuploidy (Kevers, 1999)
Nuclei with large irregular shapes and multiple nucleoli (Häsler *et al.*, 2003)	Hyperhydricity (Crèvecoeur *et al.*, 1987)	Reduced cell-cell adhesion (Liners *et al.*, 1994)
	Permanent oxidative stress (Arbillot *et al.*, 1991)	Increased susceptibility to necrosis (Kevers *et al.*, 1995)

Table 5.1: Summary of investigations on the fully habituated cell line over twenty-five years. Noting that all above morphological, biochemical and physiological characteristics are also found in animal metastases.

Profiles previously (1992-1999) constructed for the biochemical and metabolic attributes of the lines suggest that some major disturbances in both primary and secondary pathways may be occurring in these unique cultures. Much of this work was undertaken by Gaspar and colleagues. Polyamines were found to accumulate in the fully habituated, cancerous cell lines as a result of a disturbance in the Shemin pathway, which is the pathway which deals with the synthesis of haem products, including catalase, peroxidase and chlorophyll pigments (Gaspar, 1999b). Deficiencies in the tetrapyrrole containing compounds such as catalase, peroxidase and chlorophyll were all observed in the fully habituated cell lines identified by Hagège *et al.* (1992b), Gaspar *et al.* (1999) and Bisbis *et al.* (1994). In this current study levels of tetrapyrrole compounds (chlorophyll, catalase and peroxidase) were

found to be as follows: The normal cell line still contained chlorophyll as seen by the colour of the callus, however the intensity of pigmentation was lower than might have been expected and colour did not intensify over the subculture period; however, as chlorophyll quantification was not determined, any quantitative changes in chlorophyll intensity were not confirmed.

Catalase activity was higher in the normal cell line compared to the fully habituated cell lines (N1 and N3), however there was less difference in activity between the normal and fully habituated cell lines compared to the results recorded by Hagège *et al.* (1992b), suggesting that there was a further decrease in catalase activity in the normal cell line since 1992. Activity of catalase was also measured in the habituated organogenic cell line and the results demonstrated a decrease in catalase activity as the neoplastic state progressed in the four *B. vulgaris* cell lines.

Peroxidase activity is known to be low in non-organogenic cell lines and has been previously shown to be very low in the fully habituated non-organogenic cell lines (N1 and N3) (Hagège *et al.*, 1992c and Bisbis *et al.*, 1994). It is understood that normal nitrogen metabolism is disturbed in habituated tissues, which results in an accumulation of benzoic acid derivatives and disturbs the normal Shemin pathway. This results in an overall decrease in the level of porphyrin compounds and a corresponding increase in polyamines. Peroxidase activity is also linked with lignin production, thus the higher the lignin content the higher the peroxidase activity (Halliwell, 1981c and Joersbo *et al.*, 1989). The present study revealed that peroxidase activity was only significantly higher in the habituated organogenic cell line (cell line HO); this cell line is the only one, which would be expected to contain a

significant amount of lignin, due to its organogenic capacity, which may explain this observation.

There was no significant difference in peroxidase activity between the normal and the fully habituated cell lines, as these cell lines are all callus cultures, they will have less lignin than the organogenic cell line, and this may possibly explain the lower peroxidase levels. It may also suggest that the normal cell line has suffered a decrease in peroxidase levels as a result of a disturbed Shemin pathway. To confirm that this is the case the re-evaluation of polyamine levels in the normal cell line would be required. This was not carried out during this study but may provide more valuable information to suggest whether the normal cell line is in fact entering spontaneous neoplastic progression, as disturbances in polyamine metabolism are often associated with habituated tissues (Gaspar, 1999b). What exactly causes the disruption in nitrogen metabolism has still not been clearly established, however any change in metabolism is normally initiated by some form of signalling molecule, and there is some speculation that reactive oxygen species could be involved (Le Dily *et al.*, 1993). A shift in the Shemin pathway would also explain the low catalase activity, however the low levels found in all the *B. vulgaris* cell lines observed during the current study, suggest that catalase activity may also be lowered further by ROS deactivation as a result of its vulnerable structure (Boon, 2001).

Previous studies on superoxide activity in these cell lines have been carried out by Hagège *et al.* (1992b) who determined the activity of "total" SODs and found that the level of total SOD was significantly higher in the fully habituated cell lines compared to the normal (non-habituated) cell line. During this current study, where only Cu,Zn-

SOD activity was measured, (the major SOD isomer in most plants), the data shows that in all four cell lines there was no detectable Cu,Zn-SOD. There has been no previous data published on this observation and the reason for the apparent deactivation or loss of expression of SOD in these *B. vulgaris* cell lines is not clearly understood. Some possible explanations are that the loss of Cu,Zn-SOD activity may be connected to an increase in the number of deficient chloroplasts. But if this were the case then some Cu, Zn-SOD activity would have been expected in the normal cell line, where it is assumed the chloroplasts are less damaged, as chlorophyll production is still visible. This leaves other possibilities: either a complete deactivation of Cu,Zn-SOD has occurred, as a result of a change in the normal gene expression because of DNA mutation or a programmed change through somatic variation, or these cell lines never contained any Cu,Zn-SOD in the first place, which is unlikely as almost all plant cells are thought to contain Cu,Zn-SOD. To elaborate further on the activity of the various forms of superoxide dismutase it would be necessary to carry out analysis of each isoform separately. The most accurate method for doing this would be to analyse the expression of each of the proteins involved in each isoform using Western blotting techniques. Thus, further studies are recommended at the molecular and post-translation level to elucidate why these cell lines lack the main SOD isomer and to evaluate the possible presence of other isomers.

Glutathione content was last determined in these cell lines by Hagège *et al.* (1992b), who showed that the glutathione ratio in the normal cell line was approximately 0.9, which means that 90% of the total glutathione was in the reduced form. The fully habituated cell lines were shown to have a ratio of approximately 0.8, thus 80% of the total glutathione was in the reduced form. In normal healthy cells the reduced

glutathione percentage is thought to be at least 90% (Carvalho and Amâncio, 2002). Therefore, the results of Hagège *et al.* (1992b) suggest that the "normal" cell line had a normal glutathione ratio, whereas the fully habituated cell line had a slightly lowered ratio, and one just below that of optimum cellular levels. Glutathione levels measured during this current study showed dramatic changes in the glutathione ratio, particularly in the fully habituated cell lines (N1 and N3). The ratio in the "normal" cell line had dropped to 0.7, therefore the percentage of reduced glutathione had fallen by a further 20% since the study performed in 1992; the fully habituated cell lines showed an even more dramatic drop in the glutathione ratio and the ratio was now between 0.2-0.4, which is a 40-60% decrease in reduced glutathione levels, since the previous evaluation (1992).

Such a decrease in GSH levels will have an impact on the overall redox potential as reduced glutathione reacts as a biological buffer and facilitates many biochemical reactions, including many antioxidant and detoxification processes. Therefore a reduction in the redox potential will undoubtedly decrease the antioxidant status of the cell line and will shift the antioxidant / pro-oxidant equilibrium towards the pro-oxidant side. The most likely reason for glutathione depletion is an increase in ROS, accompanied by a decrease in activity of some key antioxidant enzymes which then predisposes cells to secondary, oxidative stress manifested as lipid peroxidation and mutational DNA changes with further enzyme deactivation being the most likely outcome. Moreover, an increase in glutathione transferases involved in the detoxification of aldehydic products of lipid peroxidation may also further compromise the overall GSH status of the lines.

Glutathione reductase is involved in the recycling of glutathione and in previous studies (Hagège *et al.*, 1992b) the activity of glutathione reductase was shown to be significantly higher in the fully habituated cell lines, probably in response to the slight decrease in reduced glutathione concentration. The present study suggests that the level of glutathione reductase activity has decreased since 1992 in the fully habituated cell lines and there are no longer any significant differences in activity between the normal and fully habituated cell lines. Factors that contribute to the loss of glutathione reductase activity include loss of redox control; accumulation of glutathione conjugates resulting from a higher glutathione S-transferase activity, without the increase in conjugate removal. The role of glutathione reductase is to recycle oxidised glutathione (GSSG) back to reduced glutathione (GSH) in a NADPH dependent recycling reaction; therefore the reduction in glutathione activity may also be caused by a reduction in available NADPH.

The activity of glutathione S-transferase was determined during this study and it was found that activity in the fully habituated cell lines (N1 and N3) was very high compared to the habituated organogenic and the normal cell line. The significantly higher expression of glutathione S-transferase activity in the fully habituated cell lines suggests that lipid peroxidation, that produces toxic products including MDA and 4-HNE, is occurring to a much higher extent in the cancerous (fully habituated) cell lines. The connection between higher lipid peroxidation, increased MDA and elevated glutathione S-transferase expression has been well described in previous research in mammalian cells (Fukuda *et al.*, 1997), however this if the first time this same connection has been elaborated in plant cancers.

Sulphydryl groups, in particular protein bound –SH groups, have been previously used as an indicator of oxidative stress (Korotchkina *et al.*, 2001 and Chevrier, 1988). The levels of protein bound –SH groups have not been characterised in these cell lines previously and during this present study their levels were found to be almost completely depleted in the fully habituated (cancerous) cell lines. This is a strong indication that there is extensive enzyme, protein and membrane damage occurring (via the oxidation of the attached –SH groups), which will cause widespread enzyme deactivation and will leave the membranes highly prone to lipid peroxidation and destabilisation.

Arbillot *et al.* (1991) demonstrated that the fatty acid and lipid composition in membranes differed between the normal and fully habituated cell lines. They showed that the fatty acid composition in the normal callus was considerably more stable than the composition in the fully habituated cell line and the lipid composition in the normal cell line had a higher degree of unsaturation, which may be related to the diminished sulphydryl groups. The degree of unsaturation in the fully habituated cell lines diminished as the subculture period progressed, particularly after day 14, which has been associated with the extensive necrosis observed in these cell lines. Arbillot *et al.* (1991) reported that the membranes of the fully habituated cell lines were abnormally permeable resulting in the loss of membrane integrity and an increased risk of peroxidation. This atypical lipid and fatty acid composition corresponded with an increase in MDA levels indicating that lipid peroxidation was occurring as a result of the changes in fatty acid and lipid composition (Arbillot *et al.*, 1991).

The integrity of the cell membranes and the extent of membrane damage as a result of lipid peroxidation were determined during this study by measuring the levels of specific lipid peroxidation products. The levels of lipid hydroperoxides, conjugated dienes, TBA reactive substances and Schiff's bases were determined and were all found to be significantly higher in the fully habituated cell lines.

The data collected from this current study highlights the link between lowered antioxidant activities and increased lipid peroxidation products particularly in the cell lines that have lost totipotency, suggesting a correlation between the antioxidant status, extent of lipid peroxidation and the totipotency of the cultures. This agrees with the findings of Benson and Roubelakis-Angelakis (1994) where it was shown in grapevine cultures that dedifferentiation stages of tissue culture was associated with a considerable rise in TBA reactive substances and Schiff's bases, with a corresponding decrease in SOD activity. In several other cases the link between antioxidant status and culture totipotency has also been observed (Cutler *et al.*, 1989; Earnshaw and Johnson, 1985 and Benson *et al.*, 1992b). The observed increases in specific lipid peroxidation products and decreases in key antioxidants and redox potential could again be harnessed as biological markers of potential decreases in regenerative capacity.

5.2.2 Potential consequences of oxidative stress: A mutational basis of plant cancers involving ROS?

The mutational basis of cancer is a very important consideration as it is very well established that the process is caused mainly by genetic changes (mutations) in the

DNA of somatic cells (Halliwell and Gutteridge 1999). Cancer is initiated from one cell in a tissue undergoing a mutation, which gives that cell a growth advantage over other cells. When this cell divides the mutation is passed onto subsequent generations of daughter cells. The accumulation of mutations causes the cells to become more aggressive which leads eventually to the production of true cancer cells, which proliferate uncontrollably. Mutations can occur as genetic changes, such as alterations in chromosome numbers (polyploidy and aneuploidy), changes in chromosome structure, including translocations, duplications and deletions and modifications in DNA base sequences by mutations or epigenetic changes including DNA amplification and methylation (Schaeffer, 1990). The types of genes that are mutated in cancerous cells are often referred to as oncogenes or tumour suppressor genes. Oncogenes, once mutated, stimulate abnormal cell division and act in a dominant fashion within the cell. Tumour suppressor genes prevent uncontrolled cell division, however if mutated can lead to uncontrolled proliferation. The mutation of DNA repair genes is also thought to be involved in the formation of cancer, as inactivation of these genes impairs the ability to repair mutations, which will accelerate the transformation of cells (Halliwell and Gutteridge, 1999).

Numerous ROS are capable of causing DNA damage (Termini, 2000 and Cadet *et al.*, 1995) and lead to structural alterations in DNA, which can result in conformational changes causing a decrease in DNA repair as the DNA repair enzymes do not recognise the structure of the damaged DNA. ROS can also modulate stress-induced proteins and genes, leading to a change in the regulatory processes that control cell growth, differentiation, and cell death processes (necrosis and programmed cell death) (Burdon, 1989; Kairong *et al.*, 2002 and Jacobson, 1996). Direct DNA base damage

by ROS can induce physical chemical changes in DNA, such as modified hydrogen bonding and blocked replication that results in DNA mutation.

In the case of *in vitro* plant cultures somaclonal variation is the main process identified with genetic instability (Scowcroft and Larkin 1982). The causes of somaclonal variation are numerous and the processes by which they are initiated still remain difficult to ascertain due to the complexity of the process which is manifested as phenotypic variation brought about by either genetic or epigenic mutations. As it is known that somaclonal variation occurs during *in vitro* manipulations it is possible that this type of genetic variation has contributed to the initial habituation observed in the fully habituated cell lines. Thus, if Häsler's (2005) theory of spontaneous habituation occurring in the normal cell line is true then somatic variation may be one of the causes implemented by the length of time these cultures have been maintained in culture. Repetitive accumulation of somaclonal variation will hence lead to gradual drift in phenotypic characters away from the norm and as these progresses they may eventually lead to neoplastic progression and to fully cancerous (habituated) cells. However, it is important to consider that somaclonal variation is progressive and many somaclonal variations are heritable and under these circumstances cells do not lose totipotency. However, when cells become fully habituated they lose totipotency, and at this stage somaclonal variation is no longer an issue for regeneration.

Somaclonal variation frequently manifests itself as random mutations, which can result in changes in chromosome numbers (polyploidy and aneuploidy), changes in chromosome structure (including translocations, duplications and deletions) and changes in DNA base sequences by mutations (Schaeffer, 1990). Epigenetic changes

include DNA amplification and methylation (Schaeffer, 1990). As somaclonal variation occurs to a higher extent in callus cultures, particularly those on media containing 2,4-D (Saieed *et al.*, 1991) this is an important consideration in tissue culture manipulations. Large deletions in rice chloroplast genomes have been previously shown to cause a loss of totipotency (Abe *et al.*, 2002). The normal *B. vulgaris* cell line (N) has been maintained in medium containing 2,4-D for the last twenty-four years, therefore somaclonal variation may contribute to the changes observed in this cell line during this study. The genetic variation in the *B. vulgaris* cell lines has not been studied in detail and it may be advantageous to carry out detailed research to investigate variation in the mitochondrial and chloroplast genomes, using southern blotting techniques and a number of chloroplast and mitochondrial DNA probes.

5.2.3 Do free radicals and ROS mediated reactions play a role in plant cancers?

The presented data collected on the *B. vulgaris* cell lines show that in all respects that the fully habituated cell lines exhibit the most disturbed antioxidant / pro-oxidant status. Enzyme deactivation, antioxidant depletion and increased lipid peroxidation are all directly related to oxidative stress. The evidence therefore suggests that these cell lines not only show signs of an antioxidant / pro-oxidant imbalance but in both the normal and fully habituated cell lines there is evidence to suggest that the cells have undergone further loss in antioxidant potential. The habituated organogenic cell line, although more organised, shows several morphological abnormalities and also displayed significant decreases in antioxidant activity.

Levels of lipid peroxidation were much higher in the fully habituated cell lines; however there was evidence of lipid peroxidation to a much lower extent in the habituated organogenic and normal (non-habituated) cell lines. Increased lipid peroxidation and decreased antioxidants are classic signs of oxidative stress (Bremner *et al.*, 1997; Benson *et al.*, 1992 and Benson and Roubelakis-Angelakis, 1994) therefore it is reasonable to conclude that oxidative stress is elevated in fully habituated cells and may be the cause of the irreversible phenotypic changes and permanent changes in phenotype. The theory posed by Häsler *et al.* (2004) suggesting that the normal, previously thought to be un-habituated cells, may be undergoing neoplastic progressions leading to a habituated state could not be confirmed directly in this study. However, the evidence is in support to some extent and future progress now requires a more detailed assessment of oxidative status in conjunction with molecular studies directed at gene expression and silencing. This was considered in the present study, through the application of investigations into DNA methylation. However, for technical issues related to low levels of DNA in extractions it was not possible to pursue this work further within the time frame of the study. Nevertheless, for future studies it is recommended that these molecular approaches be revisited.

5.3 Applications of the research in plant biotechnology: markers of totipotency and antioxidant treatments

More conclusive evidence for linking cancer and ROS in these *in vitro* cultures resides in the fact that the antioxidant status of the "normal" cells has definitely decreased and may therefore contribute to the initiation of their neoplastic progression. Further in-depth studies are necessary to determine changes in hormone dependency / sensitivity

and the other physiological, biochemical and genetic variations taking place and this would provide much more information on the "normal" *B. vulgaris* cell line. The future study of this cell line may also identify some of the initial key changes that occur during neoplastic progression and habituation. This would provide an important practical application in the identification of stress markers for the loss of totipotency. To be able to identify diagnostically problems in plant tissue cultures and including those impacted by ageing would be of great benefit to plant biotechnologists. Therefore the identification of oxidative stress markers of habituated conditions and neoplasia has important applications. For example the reduction of the glutathione ratio may be a marker of oxidative stress, which is potentially a key contributor to the loss of redox control and loss of totipotency.

In addition to monitoring the GSH ratio it may also possible to monitor the levels of lipid peroxidation markers, MDA and 4-HNE as they have been previously been reported to be different in embryogenic and non-embryogenic cell lines (Adams *et al.*, 1999) and this may prove very useful if a sensitive method can be successfully applied to aged cultures. The loss of key antioxidants such as Cu,Zn-SOD may highlight some important changes in aged and neoplastic cell lines, however the cause of the apparent deactivation needs to be investigated to determine whether is could be used as a biochemical marker.

Potential areas of use also include genetic transformation studies for which dedifferentiated growth is an intermediary stage and morphogenetic regeneration can be compromised by rapid *in vitro* ageing, *in vitro* conservation and micropropagation.

A further approach to understanding the *in vitro* stress physiology of plants is to address their impacts in relation to *in vivo* adaptations and such an approach may help develop improved tissue culture methodologies. Plants possess a vast array of biochemical stress defences and these are clearly defined for most stresses, e.g. temperature, drought, and pathogen attack. The responses involve stress perception, signalling and the activation of transcription factors and genetic reprogramming. However, tissue culture is an unnatural process for plants and exposes their cells and developmental pathways to stresses and stress combinations that they may not naturally encounter in nature. Their ability to overcome many stresses presented by *in vitro* manipulations demonstrates their plasticity and the ability of the genome to detect and respond to novel stresses. For example in the shift from autotrophic to heterotrophic metabolism and in the application of potent plant growth regulators, such as 2,4-D that was initially developed as a herbicide.

A greater understanding of these stresses may make it possible to apply corrective procedures that could reduce their impacts. The first stress that plant tissues in culture are exposed to is the oxidative stress resulting from severe wounding at excision point of the explant. This is associated with activation of the cell cycle, cell dedifferentiation and expression of totipotency. It is also associated, in some genotypes, with genome instability (somaclonal variation), (Bhat and Srinivasan 2002). Following establishment in culture, the plant tissues may be stressed by the high salt or unbalanced mineral composition of the medium and by the effects of plant growth regulators in the medium. Collecting more data about the process of cellular ageing and habituation and defining which antioxidants may be affected and determining what is the prime cellular target will aid in determining what the

accelerators of stress are and whether the process could be reversed. The development of new culture techniques, which reduce stress in the "tissue culture habitat", will be important for studying ageing and habituation.

Decreases in oxidative stress through tissue culture manipulations have been achieved by several different methods, for example, in new and improved tissue culture vessels that have been found to improve gas exchange and humidity levels (Mensuali-Sodi *et al.*, 1993), addition of antioxidants such as ascorbate and vitamin E (Ortiz and Shea, 2004), exogenously added polyamines (Tang *et al.*, 2004) and exogenously added calcium (Li *et al.*, 2003), (see section 1.5 for more details on other tissue culture improvements).

The progressive loss of totipotency in *in vitro* tissue culture is thus almost certainly connected with the ageing process and neoplastic progressions, however the exact mechanism that causes the loss of totipotency has not yet been pinpointed however the evidence collected during this current study and several other studies (Gaspar, 2000; Benson, 2000a; Benson, 1992b; Benson, 1997 and Gaspar, 1998) suggests that oxidative stress is a major contributor. Thus this thesis study provides a detailed insight into the antioxidant status of both aged and cancerous cell lines. This poses the question as to whether the information can be used to overcome recalcitrance and loss of totipotency through the application of exogenous antioxidants? There has already been a significant amount of research carried out on improving current tissue culture systems, such as the addition of somatically active compounds such as mannitol (Faure *et al.*, 1998), and the use of activated charcoal in the medium (Wang, 1976).

There are four main stages in *in vitro* manipulation that have been classified by Benson *et al.,* (2000b) as follows: (A) tissue culture initiation, (B) morphogenic transition, (C) medium term proliferation (1-6months) and (D) long term proliferation. Each stage is associated with stress however the physical stress and the manifested outcome may be different in each stage (Benson *et al.*, 1992 and Benson and Roubelakis-Angelakis 1994). Therefore in terms of antioxidant applications each stage of the tissue culture process should be assessed in terms of its oxidative stress status and protectants applied in the context of this knowledge. Tissue culture media contains several components that can cause pro-oxidant reactions, in particular plant growth regulators and metal cations. Therefore a useful starting point would be reduce the level of pro-oxidants produced in the medium, before antioxidant applications are applied to the cultures. Specific antioxidants could be added to the tissue culture medium relatively easily, however it is important to consider that the addition of just one antioxidant may not be sufficient, as many antioxidants are interlinked and are tightly regulated redox reactions and the addition of other redox components may be required. Also the interplay between the antioxidants may also mean that the addition of one antioxidant may have adverse effects on another antioxidant.

With respect to this current study in both the aged and habituated cultures the GSH/GSSG redox ratio was highly disturbed, suggesting chronic oxidative stress. Therefore it may be possible to add exogenous glutathione to the tissue cultures as a means of re-establishing the redox ratio, which may in turn reduce oxidative stress in these cell lines. However the addition of GSH alone may have no effect without the addition of the other substrates involved in the antioxidant pathway, these include ASC and NADPH. Managing stress in *in vitro* cultures can result in reduction of the

harmful aspects and exploitation of the beneficial aspects and may also aid in the culture of previously recalcitrant species (Benson, 2000). This area of tissue culture improvement would greatly benefit from more in depth research.

5.4 Future recommendations

This study has highlighted many similarities between cancerous plant cells and cancerous mammalian cells; there is therefore an opportunity for further research to discover the differences and similarities of ageing and cancer progression in plant and mammalian cell culture systems. A more detailed cytological investigation is required to determine the relationship between the cytological and biochemical changes resulting from *in vitro* ageing and neoplastic progression (habituation). More detailed studies of the effects of plant growth regulators and the loss of sensitivity to them would be an appropriate route to take.

One of the key areas for further study pertains to the impacts and consequences of habituation and *in vitro* ageing for genetic stability. Somaclonal variation is a major issue in plant biotechnology programmes and understanding the basis of this complex phenomenon is very important. From a molecular point of view, changes in gene expression in these cell lines have not been examined in any great detail and both cell lines would benefit from further investigation of genetic variation. It would be particularly interesting in the future to study the genetic expression patterns of superoxide dismutase isoforms (Cu,ZN-SOD, Mn-SOD and Fe-SOD), as this may provide an explanation for the complete deactivation of Cu,Zn-SOD in the habituated cell lines and deactivation in the some of the aged cell lines, such as is observed in the

cell lines and deactivation in the some of the aged cell lines, such as is observed in the white *G. max* cell line (SW) and the non-habituated aged *B. vulgaris* cell line (N).

Catalase activity is also very low in all these cell lines and a study of the genetic expression patterns of this enzyme may clarify whether gene expression is decreased or whether ROS deactivation of the enzyme is the cause of catalase demise. The decrease in available glutathione was very apparent in all the aged and habituated cell lines and as glutathione is so important it would be extremely beneficial to identify what caused its decrease. Therefore, it would be advantageous to study the expression patterns of the enzymes involved in both the synthesis and recycling processes of glutathione. In the habituated *B. vulgaris* cell lines there is a severe decrease in the level of protein bound sulphydryl groups; therefore it would also be valuable to examine the expression patterns of the enzymes involved in replacing the protein bound sulphydryls. Future studies such as these would not only assist in the understanding of *in vitro* stress and ageing but would have wider applications in the more general understanding of plant ageing *in vivo*.

The study of plant cancer (habituation) is a relatively new area of research compared to the mammalian cancer field. This project for the first time has provided and biochemical analysis of the antioxidant status and the subsequent effects of the status in a rare set of cancerous plant cultures. Although there will always remain a major difference between the two systems, whereby mammalian cancers are far more complex, due to the fact that they can become malignant and can be dispersed systemically in mammalian systems, whereas plant cancers appear only to be localised and are non-malignant. The fundamental similarities between cancer cells in the two

kingdoms do exist, the data collected during this study and previous studies show that there are some striking similarities between the biochemical, cytological and morphological characteristics of cancer cells in plants and in mammalian cells. These similarities should be explored further as the there will be valuable insights related to plant cancers, which may prove very useful in the study of mammalian cancers. There is most importantly a real potential for these kingdoms to share a number of key cancer "markers" based on the information collected to date.

6.0 CHAPTER SIX: APPENDICES

6.1 Stock solutions and media components

- ***Beta vulgaris* stock solutions and media instructions**

Solution A (g/L)

$NaH_2PO_4.2H_2O$	2.5
KCl	6.0
$(NH_4)_2SO_4$	4.0
$MgSO_4.7H_2O$	5.0
KNO3	20
$CaCl_2.2H_2O$	3

Solution B (g/L)

H_3BO_3	0.62
$MnSO_4.H_2O$	0.168
$ZnSO_4.7H_2O$	0.1060
KI	0.1583
$Na_2MoO_4.2H_2O$	0.025
$CuSO_4.5H_2O$	0.0025
$CoCl_2.6H_2O$	0.0025

Solution C (g/L)

FeNa EDTA	2.02

Solution D (g/L)

Inositol	0.1
Thiamine	0.01
Pyridoxine	0.001
Nic Acid	0.001

Solution E (g/L)

H_3BO_3	2.0

For 1 litre of *Beta vulgaris* medium:

Solution A 100ml

Solution B 1ml

Solution C 20ml

Solution D 10ml

Solution E 5ml

Hormones

BAP 0.1mg/L

2,4D 0.1mg/L

Carbon source

Sucrose 30g/L

Agar

Plant Agar 7.0g/L

pH 6.0

- ***Glycine max* stock solutions and media instructions**

Solution A (g/L)

KH_2PO_4	6.0
KNO_3	20.0
NH_4NO_3	20.0
$Ca(NO_3)_2.4H_2O$	10.0
$MgSO_4.7H_2O$	1.44
KCl	1.30
$MnSO_4$	0.28

Solution B (g/L)

NaFe EDTA	0.264
$ZnSO_4$	0.076
H_3BO_3	0.032
KI	0.016
$Cu(NO_3)_2.3H_2O$	0.007
$(NH_4)_6Mo_2O_4$	0.002

Solution C (g/L)

Myo Inositol	4.0
Nicotinic Acid	0.080
Pyridoxine HCl	0.032
Thiamine	0.032

For 1 litre of *Glycine max* medium:

Solution A 50ml

Solution B 50ml

Solution C 50ml

Hormones

NAA 2mg/L

BAP 1mg/L

Carbon source

Sucrose 30g/L

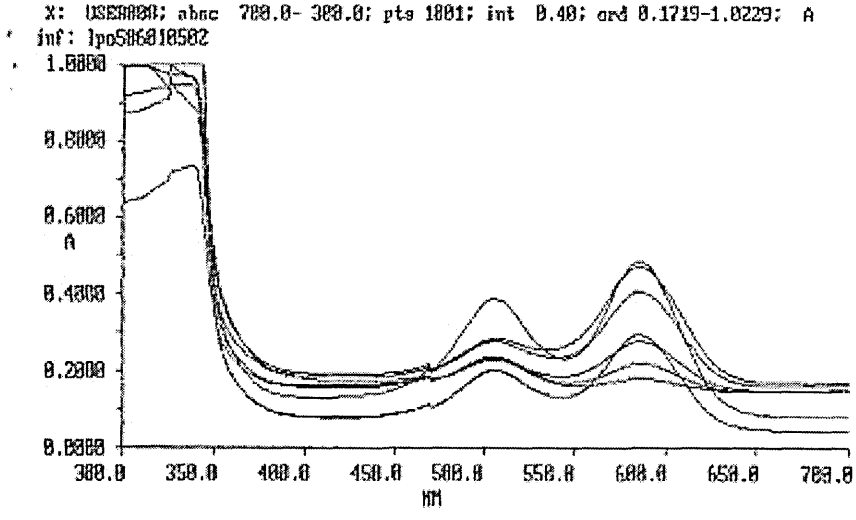
Agar

Plant Agar 7g/L

pH 5.8

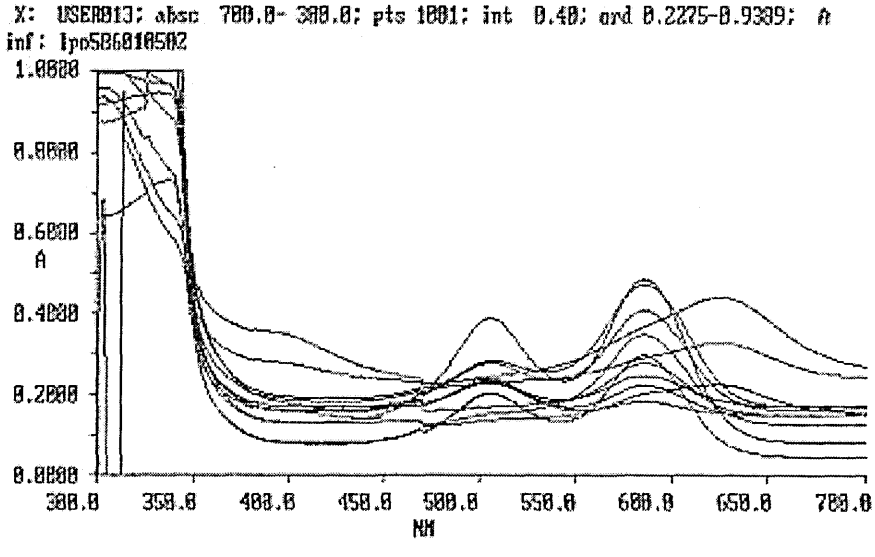
*add filter sterilised hormones after autoclaving.

6.2 Scans from LPO-586 assay



1. Standards scan (10μM-60μM)

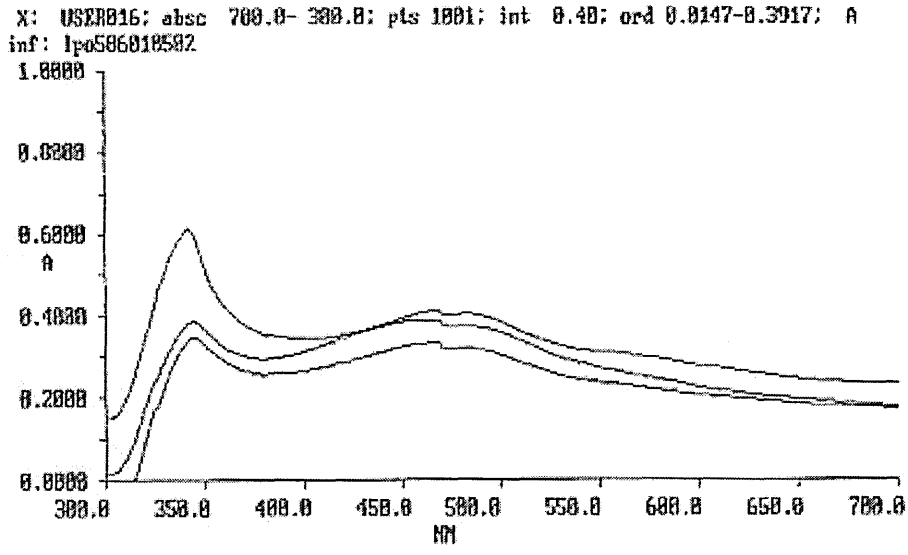
   Peaks observed at 505nm and 586nm



2. Standards scan (10-100μM)

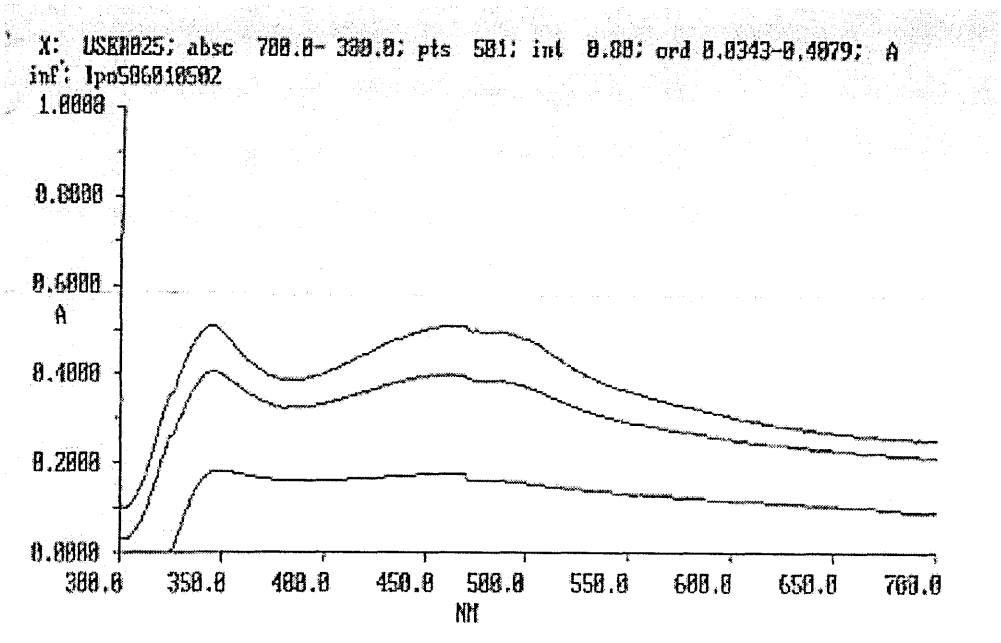
   Peaks observed at 505nm, 586nm and above 60μM peak appears around 640nm

   (suggests peak shifting above 60μM standard concentration)



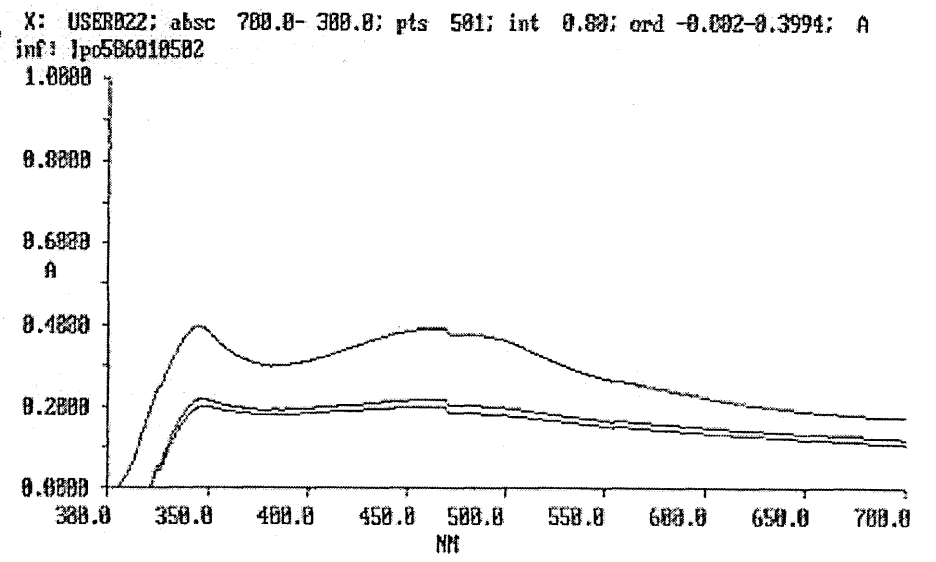
3. Sample *Beta vulgaris* N (replicates A,B & C)

   Peaks observed at 469nm



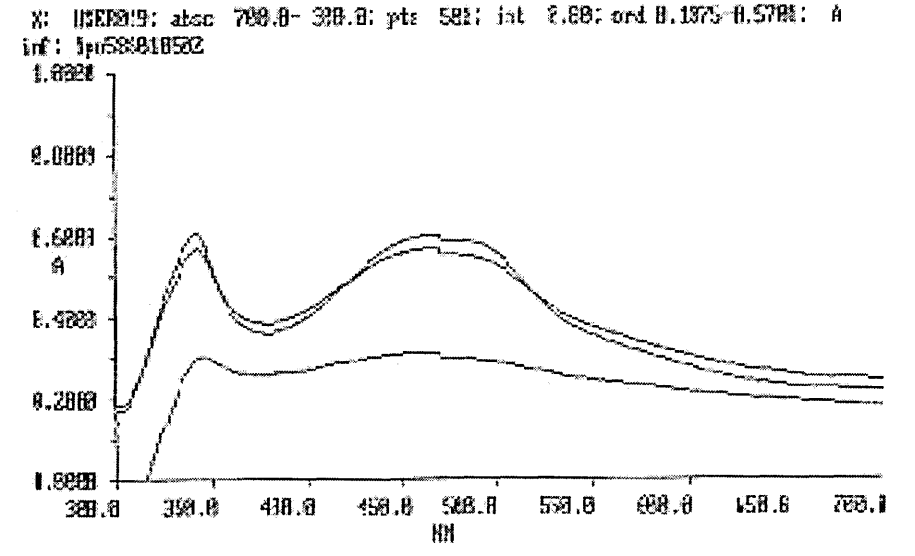
4. Sample *Beta vulgaris* HO (replicates A, B & C)

   Peaks observed at 469nm.



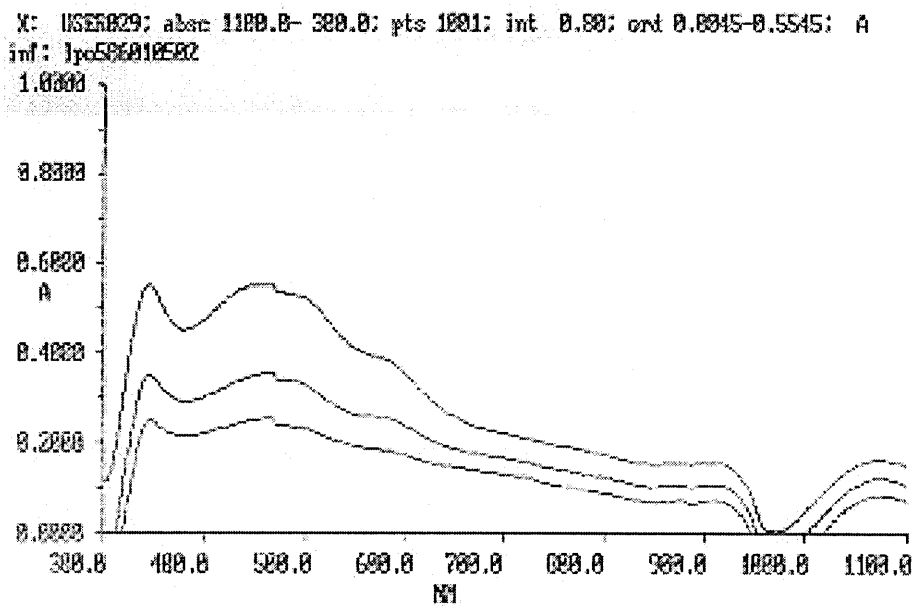
5. Sample *Beta vulgaris* N1 (replicates A, B & C)

   Peaks observed at 469nm



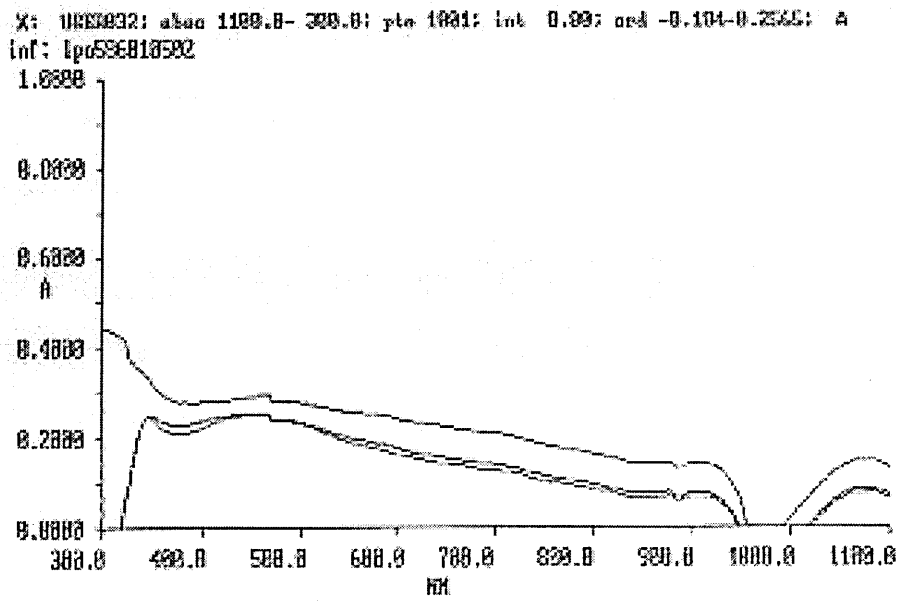
6. Sample *Beta vulgaris* N3

   Peaks observed at 469nm



7. Sample *Glycine max* SW (replicates A,B & C)

   Peaks observed at 469nm



8. Sample *Glycine max* SG (replicates A, B & C)

   Peaks observed at 469nm

7.0 CHAPTER SEVEN: LIST OF REFERENCES

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